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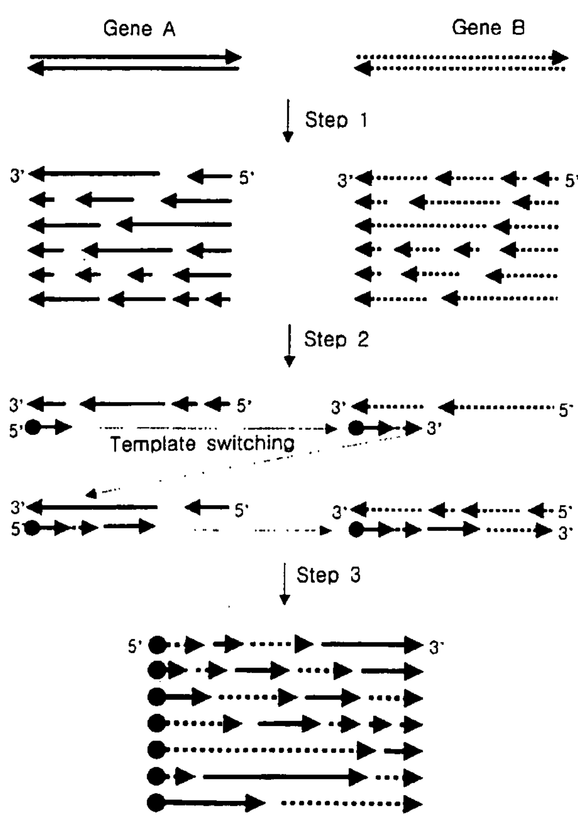

(57) Abstract: The present invention relates to a method for producing a recombinant polynucleotides comprising the steps of generating a pool of unidirectional single-stranded polynucleotide fragments from two or more homologous double-stranded polynucleotides, conducting a polymerization process comprising multi-cyclic extension reactions using the unidirectional single-stranded polynucleotide fragments as templates and specific oligonucleotides as primers to obtain recombinant polynucleotides, and conducting a polymerase chain reaction using at least one primer to amplify the recombinant polynucleotides; and a method for constructing a recombinant DNA library comprising the steps of inserting the recombinant polynucleotide prepared by the above method into a vector and transforming an expression cell with said vector containing the recombinant polynucleotide to obtain a plurality of mutant clones. The method of the present invention can be used for in vitro recombination of homologous polynucleotides and the directed molecular evolution.

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METHOD FOR GENERATING RECOMBINANT DNA LIBRARY USING UNIDIRECTIONAL SINGLE-STRANDED DNA FRAGMENTS

FIELD OF THE INVENTION

The present invention relates to a method for the production of a pool of recombinant DNA encoding mutant proteins and a recombinant DNA library comprising same, which allows the directed evolution of proteins by *in vitro* recombination.

BACKGROUND OF THE INVENTION

Genetic information is eventually decoded into a protein which performs most of the vital functions in living organisms. As one of important biological macromolecules, protein not only serves as a component of cells but also participates in all the biochemical reactions with a high specificity.

The function of protein comprised of 20 kinds of amino acids is determined by the structure which is divided into four levels; primary, secondary, tertiary and quaternary structures. Since the primary structure of protein, i.e., amino acid sequence, especially contains the information regarding the shape and the function thereof, the whole structure or function of the protein can be changed even by a mutation in one amino acid residue (Shao, Z. and Arnold F.H., *Curr. Opin. Struct. Biol.* 6:513-518, 1996).

The diversity of organism reflects the diversity of genetic information encoded in DNA or RNA. In nature, the genetic information is changed slowly and continuously by a natural evolution process comprising mutation, sexual reproduction and natural selection. For example, during meiosis in sexual reproduction, homologous chromosomes derived from two individuals might exchange or reassemble their genetic materials through homologous recombination. Such reassembly of the DNA provides more chances for living organisms to expedite an evolution. However, it takes long time for

this type of evolution to occur in natural environment, partly due to its strong dependency on fortuity. Therefore, there have been many efforts to obtain, in a short period of time, a gene evolved for the desired purpose and a mutant protein by *in vitro* mutagenesis in combination with an appropriate screening method(Eigen, M., *Naturwissenschaften* 58:465-523, 1971; Bradt, R.M., *Nature* 317:804-806, 1985; Pal, K.F., *Bio. Cybern.* 69:539-546, 1993).

Current method in widespread use for creating mutant proteins is site-directed mutagenesis(Sambrook, J. *et al.*, *Molecular Cloning* 2nd, Cold Spring Harbor Lab Press, 1989). This method replaces nucleotides of desired site with a synthetically mutagenized oligonucleotide. However, there are limitations of the method in that it requires exact information on the amino acid sequence and the function of the site to be mutagenized in proteins. As another method for creating mutant proteins in a recombinant DNA library format, error-prone polymerase chain reaction(error-prone PCR) is used widely(Leung, D.W. *et al., Technique* 1:11-15, 1989; Caldwell, R.C. *et al., PCR Methods and Applications* 2:28-33, 1992). Error-prone PCR can be used for constructing a mutant DNA library of a gene by controlling the polymerization conditions to decrease the fidelity of polymerase. However, the error-prone PCR suffers from a low processibility of the polymerase, which limits the practical applications of the method for average-sized gene. Another limitation of error-prone PCR is that the frequency of co-occurrence of a plurality of mutations within a short-length region of DNA is too low for multiple mutations to be introduced.

To overcome said shortcomings of these methods, various methods for constructing a mutant DNA library from the mixture of homologous polynucleotides have been developed. Those are DNA shuffling method of Maxygen(USP 5,605,793; 6,117,679; 6,132,970), Gene Reassembly method of Diversa(USP 5,965,408) and recombination method developed by Frances H. Arnold(USP 6,153,410).

The DNA shuffling method of Maxygen, Inc.(USP 5,605,793; 6,117,679 and 6,132,970; Stemmer, W. P. C., *Nature*, *370*: 389-391, 1994; Stemmer, W. P. C., *Proc. Natl. Acad. Sci. USA*, *91*: 10747-10751, 1994)

comprises the steps of fragmenting at least one kind of double-stranded DNAs to be shuffled and conducting polymerase chain reactions(PCR) with the combined fragments, wherein the homologous fragments from different parent DNAs are annealed with each other to form partially overlapping DNA segments and DNA synthesis occurs by employing the respective DNA fragments as a template concurrently as a primer for each other to produce a random recombinant DNA library. However, this method requires a relatively large amount of DNA for preparing DNA fragments and DNase I used in the fragmentation process has to be removed from the resulting DNA fragments in an enough purity not to disturb subsequent polymerization process. Further, the application of the method is limited by the property of the DNase I. For example, DNase I widely used for the purpose is liable to cleave a 3'-phosphodiester bond having a pyrimidine base rather than a purine base at its terminus, which is a serious obstacle to get a completely randomized pool of DNA fragments(Shao, Z. *et al., Nucleic Acids Res.* 26:681-683, 1998).

Gene Reassembly method of Diversa Corporation(USP 5,965,408) comprises the steps of synthesizing DNA fragments by polymerization process employing at least one kind of double-stranded DNAs to be shuffled as templates and conducting polymerase chain reactions(PCR) with the combined fragments to produce a random recombinant DNA library. It employs partially synthesized fragments produced by UV treatment or adduct formation on the template DNA, thus preventing a complete polymerization on the template DNA. Despite of the randomness of the constructed DNA library, there are still problems for the method of Diversa Corporation in view of mutagenic potential of used reagents and tediousness to optimize the reaction conditions for the treatment of polymerization terminating reagent to obtain the desired size of fragments. In addition, when pyrimidine bases exist contiguously on the DNA strand, UV treatment induces pyrimidine dimers such as a thymidine dimer, which makes the template DNA distorted and prevent the progress of polymerase along with the strand. As a result, polymerizations are likely to end up at the site of pyrimidine dimer, thus DNA fragments obtained having insufficient randomness.

DNA shuffling and Gene Reassembly methods are characterized in that the formation of partially overlapping DNA segments is a prerequisite step and each DNA fragment derived from starting DNAs to be shuffled serves as not only a template but a primer.

Another method proposed by Arnold, staggered extension process(StEP)(USP 6,153,410; Zhao, H. *et al., Nat. Biotechnol.* 16:258-261, 1998; Encell, L.P. *et al., Nature Biotech.* 16:234-235, 1998) involves priming template double-stranded polynucleotides with random or specific primers, conducting PCR while controlling the reaction conditions to produce, in each cycle of reactions, short DNA fragments of staggered extension from the templates, and conducting repeated PCR to accomplish the recombination between genes by template switching. In case of polymerase reaction, there exist specific sequence-specific pause sites in each of target DNAs. In this line, StEP method has a problem in that the recombinant DNA library is biased from randomness since the extension rate of DNA fragments extended from the primers differs from each other even if the primers are annealed to the same region of different template DNAs(Encell, L.P. and Loeb, L. A., *Nature Biotech.*, *16*: 234-235 (1998)). In StEP method, PCR conditions have to be strictly controlled in order to get short DNA fragments from staggered extension of primers by shortening the polymerization time and lowering the reaction temperature. Failure to maintain the desirable range of temperature (e.g., too low temperature) during PCR process in StEP method may lead to non-specific annealing and further formation of undesirable recombinants.

A method for constructing a recombinant DNA library whereby said drawbacks of the conventional methods are overcome would be powerful for the production of mutant proteins having improved properties. The present invention described herein is directed to a method of *in vitro* recombination of heterologous DNA strands, which comprises preparing unidirectional single-stranded DNA fragments, mixing the DNA fragments with specific primers, followed by polymerization and further repeating the above steps to produce a recombinant DNA library. Further advantages of the present invention will

become apparent from the following description of the invention with reference to the attached drawings.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method for producing various recombinant polynucleotides through the random recombination between two or more homologous double-stranded polynucleotides.

Another object of the present invention is to provide a method for constructing a recombinant DNA library, which comprises the steps of inserting said recombinant polynucleotides into a vector and transforming an expression cell with the resulting vector to obtain a plurality of mutant clones.

A further object of the present invention is to provide a method for identifying an improved mutant gene by screening recombinant polynucleotides having a desired functional properties from said recombinant DNA library.

In accordance with one aspect of the present invention, there is provided a method for constructing a recombinant DNA library comprising the steps of:

(a) generating a pool of unidirectional single-stranded polynucleotide fragments randomized in length from two or more starting polynucleotides to be reassembled which have regions of similarity with each other;

(b) conducting a polymerization process comprising multi-cyclic extension reactions wherein the unidirectional single-stranded polynucleotide fragments prepared by step (a) serve only as templates and specific oligonucleotides are added to the reaction mixture as primers,

the primers being extended sequentially with directionality by means of template switching to produce at least one recombinant polynucleotide, and the resulting recombinant polynucleotide being

different from the starting polynucleotides in nucleotide sequence; and

(c) conducting a polymerase chain reaction using at least one specific primer to amplify the recombinant polynucleotides prepared by step (b).

In accordance with another aspect of the present invention, there is provided a method for constructing a recombinant DNA library, comprising the steps of inserting the recombinant polynucleotide prepared by the above method into a vector; and transforming an expression cell with said vector containing the recombinant polynucleotide to obtain a plurality of mutant clones.

In accordance with a further aspect of the present invention, there is provided a method for evolving a polynucleotide toward a desired property which comprises screening recombinant polynucleotides having a desired functional properties from the recombinant DNA library constructed by the above method.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows the schematic diagram illustrating the inventive method for constructing a recombinant DNA library employing unidirectional single-stranded polynucleotide fragments as templates for the polymerase chain reaction.

Fig. 2 compares the nucleotide sequences of the chitinase genes of *Serratia liquefaciens* (*l-chi*)(SEQ ID NO: 1) and *Serratia marcescens* (*m-chi*)(SEQ ID NO: 2). The corresponding bases of the two genes different from each other are marked by small letters.

Fig. 3 displays the result of 1% agarose gel electrophoresis, wherein

lane 1 of (a) is a standard DNA size marker(23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb from the top), lane 2 of (a), *in vitro* transcription product of chitinase gene of *Serratia marcescens*, lane 3 of (a), *in vitro* transcription product of chitinase gene of *Serratia liquefaciens*; lane 1 of (b) is a standard DNA size marker(23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb from the top), and lane 2 of (b), single-stranded DNA fragments produced by reverse transcription; and lane 1 of (c) is a standard DNA size marker(23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb from the top), and lane 2 of (c), PCR products produced by employing unidirectional single-stranded DNA fragments as templates.

Fig. 4 represents the result of 1% agarose gel electrophoresis of the digestion products obtained by extracting the plasmid DNAs from 14 clones randomly selected from the recombinant DNA library prepared by the inventive method and digesting the plasmid DNAs with restriction enzymes *Not*I, *Pst*I and *Hinc*II. Lane 1 is a standard DNA size marker and lanes 2 to 15, digestion products of the plasmid DNAs from the randomly selected 14 clones.

Fig. 5 compares the nucleotide sequences of the 10 recombinant DNAs (SEQ ID NOs: 3 to 12), which are randomly selected from the recombinant DNA library produced by the method of the present invention, with those of two wild-type genes, i.e., *l-chi* gene(SEQ ID NO: 1) and *m-chi* gene(SEQ ID NO: 2).

Fig. 6 is a schematic diagram showing the constitutions of the mutant recombinant DNAs of Fig. 5 in comparison with the two wild-type genes.

Fig. 7 shows the difference in the sizes of the clear zones made by the colonies expressing the recombinant chitinase genes on LB-agar plates containing 100 μg/mℓ ampicillin and 0.5% swollen chitin, depending on the chitin decomposition capabilities of the colonies.

Fig. 8 compares the nucleotide sequence of R-24 chininase gene (SEQ ID NOs: 13) with those of two wild-type genes, i.e., *l-chi* gene(SEQ ID NO: 1) and *m-chi* gene(SEQ ID NO: 2).

Fig. 9 is a schematic diagram showing the constitution of R-24 chininase gene in comparison with the two wild-type genes.

Fig. 10 compares the nucleotide sequences of M-13 mutant(SEQ ID

NO: 15) and M-20 mutant(SEQ ID NO: 16) with that of wild-type chitosanase gene(SEQ ID NO: 14).

Fig. 11 depicts the differences in heat-stabilities of wild-type chitosanase derived from *Bacillus* sp. KCTC 0377BP, mutant M-13 and mutant M-20.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have endeavored to develop a new method for solving the problems of the prior art, and have accomplished the present invention by establishing a new method for producing a recombinant DNA library wherein a pool of various recombinant DNAs can be obtained more easily owing to the increased randomness introduced by a new principle different from those of the prior art.

The above-described DNA shuffling method of Maxygen, Inc.(US Patent Nos. 5,605,793; 6,117,679; and 6,132,970) and Gene Reassembly method of Diversa Corporation(US Patent No. 5,965,408) are commonly characterized in that the double-stranded DNA fragments obtained from more than two polynucleotides to be reassembled are converted to single stands and then annealed with each other to form partially overlapping DNA segments, and, accordingly, they are used as primers as well as templates for nucleotide extension in the polymerase chain reaction(US Patent Nos. 4,683,202 and 4,683,195) and elongated by repeating identical multi-cyclic polymerization reactions. In contrast, the method of the present invention is basically different from the prior art in that the unidirectional single-stranded polynucleotide fragments derived from two or more polynucleotides to be reassembled are used and, accordingly, no partially overlapping DNA segments are formed within the pool of single-stranded polynucleotide fragments and the unidirectional polynucleotide fragments serve only as templates; that just the oligonucleotides added as primers are elongated gradually with a directionality using the unidirectional single-stranded polynucleotide fragments as templates; and that recombination is introduced

by template switching during this PCR process. Further, unlike the Arnold's StEP method(US Patent No. 6,153,410) which employs the stringent conditions controlling temperature and reaction time to produce partially elongated DNA fragment from the double-stranded target DNA used as a template, the method of the present invention uses DNA fragments as templates and, therefore, DNA fragments elongated as long as the template DNA fragments can be obtained by employing a conventional condition of polymerization reaction. Further, it is possible to increase the randomness of recombination significantly since the inventive method is not influenced by the delayed elongation rate of polymerase at the sequence-specific pause sites.

The method of the present invention for producing mutant recombinant polynucleotides provides a method for producing a group of various recombinant genes by exchanging parts of two or more homologous genes with each other, and comprises the steps of:

(a) generating a pool of unidirectional single-stranded polynucleotide fragments randomized in length from two or more starting polynucleotides to be reassembled which have regions of similarity with each other;

(b) conducting a polymerization process comprising multi-cyclic extension reactions wherein the unidirectional single-stranded polynucleotide fragments prepared by step (a) serve only as templates and specific oligonucleotides are added to the reaction mixture as primers, the primers being extended sequentially with directionality by means of template switching to produce at least one recombinant polynucleotide, and the resulting recombinant polynucleotide being different from the starting polynucleotides in nucleotide sequence; and

(c) conducting a polymerase chain reaction using at least one specific primer to amplify the recombinant polynucleotides prepared by step (b).

In the polymerization reaction of step (b), when the partially elongated DNA fragments from specific primers are annealed with the template DNA

fragments originated from the other starting double-stranded polynucleotide in the next cycle and the polymerization reaction is progressed, then recombinant polynucleotides containing the sequences originating from the two homologous polynucleotides in a polynucleotide are resulted therefrom. By repeating such PCR cycles, it is possible to obtain various mutant recombinant polynucleotides having randomly reassembled sequences between A and B gene as shown in Fig. 1.

In addition, the present invention provides a method for constructing a recombinant DNA library, comprising the steps of inserting the recombinant polynucleotide prepared as above into a vector; and transforming an expression cell with said vector containing the recombinant polynucleotide to obtain a plurality of mutant clones.

It is possible to screen a useful gene from the recombinant DNA library constructed by the inventive method.

Accordingly, the present invention further provides a method for identifying an improved mutant gene, which comprises screening recombinant polynucleotides having a desired functional property from the recombinant DNA library constructed by the above method.

The present invention relates to a method for producing a recombinant DNA library by random recombination between two or more genetic materials. According to the present invention, it is possible to synthesize various kinds of recombinant genes by *in vitro* random recombination and to prepare a novel polypeptide having a desired property by screening a clone having a desired gene from a recombinant DNA library constructed by using the recombinant genes together with a suitable expression vector and a host cell and expressing the polypeptide therefrom.

As used herein, the term "unidirectional single-stranded DNA or polynucleotide fragments" means that the single-stranded DNA or polynucleotide fragments are not anti-parallel, but parallel to each other and, accordingly, they cannot anneal with each other via complementary hydrogen bonds even if they are mixed together. For instance, when the entire nucleotide sequence of a double-stranded DNA is as follows,

5'-AGGTCCAGTTAGCATTCGGAAAGGCCGTTTGAGAGAG-3' (SEQ ID NO: 17)

3'-TCCAGGTCAATCGTAAGCCTTTCCGGCAAACTCTCTC-5' (SEQ ID NO: 18)

the single-stranded DNAs derived therefrom such as 3'-TCCAGGTCAATCGTAAG-5'(SEQ ID NO: 19), 3'-AAACTCTCTC-5'(SEQ ID NO: 20), 3'-TTTCCGGCAAACTCTCTC-5'(SEQ ID NO: 21), 3'-CCTTTCCGGCAAACTCTCTC-5'(SEQ ID NO: 22) and 3'-TCAATCG TAAGCCTTTCCGGCAAACTCTCTC-5'(SEQ ID NO: 23) are considered to be unidirectional. Such unidirectional single-stranded DNA or polynucleotide fragments, which are employed in the method of the present invention only as templates for polymerase chain reactions, may be prepared to have various lengths depending on the sizes of the polynucleotides to be reassembled.

The term "recombinant DNA" as used herein means a chimeric DNA of a nucleotide sequence mosaic including nucleotide sequences originating from two or more polynucleotides, which are substantially homologous but not identical, in a molecule. The chimeric DNA contains a region of original nucleotide sequence and another region of mutated nucleotide sequence. Figs. 5 and 6 illustrates such recombinant DNAs synthesized by the random *in vitro* DNA recombination by the method of the present invention. Unlike the recombinant DNA naturally produced by the gene exchange due to the crossing over between homologous chromosomes in the meiosis during sexual reproduction, the recombinant DNAs of the inventive method is produced to have various nucleotide sequences in a short time by the *in vitro* random recombination between homologous DNA strands and they can be inserted into a vector and expressed in a host cell transformed by the vector. A recombinant DNA library consisting of clones containing various recombinant DNAs can be constructed and a recombinant DNA having a desired property can be screened therefrom. As discussed above, the combination of *in vitro* production of random recombinant DNA library between two or more homologous polynucleotides with a screening technique mimicking the natural selection has an advantage in that an improved gene or mutant protein having a desired property can be obtained in a short time.

As used herein, the term "homologous" means that one single-stranded

nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the nucleic acid sequences and the hybridization conditions such as temperature and salt concentration.

As used herein, the term "mutation" means changes in the sequence of a wild-type nucleic acid sequence or changes in the sequence of a peptide expressed therefrom.

As used herein, the term "DNA library" means a set of polynucleotides or recombinant DNA fragments each consisting of two or more polynucleotides and produced by random recombination. The DNA library includes: a set of polynucleotides having various nucleotide sequence; a sum of DNAs having various nucleotide sequences or cloned DNAs; or, in a broad sense, a set of clones containing said DNAs. A recombinant DNA encoding a protein having a desired property can be screened from such DNA library and used for protein expression.

More specifically, the present invention provides a method for producing recombinant polynucleotides having randomly and artificially mutated various nucleotide sequences from naturally existing or artificially prepared two or more homologous polynucleotides by the following steps. Fig. 1 illustrates this *in vitro* DNA recombination method.

Step 1: A set of unidirectional single-stranded polynucleotide fragments of random lengths are generated from two or more starting polynucleotides to be reassembled, wherein the starting polynucleotides have regions of similarity with each other(Step 1 of Fig. 1).

The starting polynucleotides for use in the present invention may have a homology of more than 50% with each other, and it is preferred to employ starting polynucleotides having homologies of more than 80%.

All of the single-stranded polynucleotide fragments produced from two or more homologous polynucleotides have identical unidirectional properties. Therefore, they are parallel to each other and, accordingly, a complementary

annealing between them through complementary hydrogen bonds cannot occur even if they are mixed together.

The unidirectional single-stranded polynucleotide fragments can be prepared by any one of conventional methods, e.g., a method producing unidirectional single-stranded polynucleotide fragments from RNA by reverse-transcription, a method for producing single-stranded polynucleotide fragments by gradual unidirectional deletion of nucleotides, a method for producing single-stranded polynucleotide fragments from complementary single-stranded polynucleotides. The single-stranded polynucleotide fragments can be prepared from RNA or single-stranded DNA beginning with random primers(Feinberg, A. P. and Vogelstein, B., *Anal. Biochem.*, *132*: 6-13 (1983)) by employing reverse transcriptase(Gerard, G. F. et al., *Mol. Biotechnol.*, *8*: 61-77 (1997)), bacteriophage T4 DNA polymerase(Nossal, N. G., *J. Biol. Chem.*, *249*: 5668-5676 (1974)), bacteriophage T7 DNA polymerase(Tabor, S. and Richardson, C. C., *J. Biol. Chem.*, *264*: 6447-6458 (1989)), Klenow enzyme(Klenow, H. and Henningsen, I., *Proc. Natl. Acad. Sci. USA*, *65*: 168 (1970)), etc. At this time, the size of single-stranded polynucleotide can be regulated by controlling the concentration of random primers or adding an appropriate concentration of dideoxynucleotides(2',3'-dideoxyadenosine 5'-triphosphate, 2',3'-dideoxyguanosine 5'-triphosphate, 2',3'-dideoxycytidine 5'-triphosphate, 2',3'-dideoxythymidine 5'-triphosphate) to the reaction mixture to obtain single-stranded polynucleotide fragments of which length is gradually elongated from the random primers. The single-stranded polynucleotide fragments having gradual unidirectional deletions of nucleotides may be obtained by employing exonucleases capable of successively digesting the nucleotides from the 5' end of a single-stranded polynucleotide.

More specifically, the unidirectional single-stranded polynucleotide fragments can be prepared by any one of the following processes:

A process comprising the steps of (i) conducting a transcription process to produce RNA from at least one starting polynucleotide; and (ii) conducting a reverse transcription process, wherein random primers are used as primers

and the RNA transcript of step (i) as a template;

A process comprising the steps of (i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme; (ii) producing a pool of double-stranded polynucleotides having unidirectional sequential deletion by treating the reaction mixture of step (i) with exonuclease III followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease III; (iii) treating the resulting double-stranded polynucleotides having a 5'-overhang with an S1 nuclease and a DNA polymerase to form a blunt end thereof; (iv) generating a new 3'-overhang to the same side which has 3'-overhang in step (i); and (v) treating the polynucleotides of step (iv) with exonuclease III to generate single-stranded polynucleotides;

A process comprising the steps of (i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme; (ii) treating the polynucleotides of step (i) with exonuclease III to generate single-stranded polynucleotides; and (iii) conducting a polymerization process on the single-stranded polynucleotides of step (ii) using random primers;

A process comprising the steps of (i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme; (ii) treating the polynucleotides of step (i) with exonuclease III to generate single-stranded polynucleotides; and (iii) producing a pool of single-stranded polynucleotides having unidirectional sequential deletion by treating the single-stranded polynucleotides of step (ii) with a single-strand specific 5'→3' exonuclease followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease.

A process comprising the steps of (i) conducting a polymerase chain reaction on the starting double-stranded polynucleotides using only one kind of oligonucleotide among forward and reverse primers; (ii) isolating the

resulting single-stranded polynucleotides from the starting double-stranded polynucleotides; and (iii) conducting a polymerization process on the single-stranded polynucleotides of step (ii) using random primers;

A process comprising the steps of (i) conducting a polymerase chain reaction on the starting double-stranded polynucleotides using only one kind of oligonucleotide among forward and reverse primers; (ii) isolating the resulting single-stranded polynucleotides from the starting double-stranded polynucleotides; and (iii) treating the single-stranded polynucleotides of step (ii) with a single-strand specific 5'→3' exonuclease followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease; and

A process for preparing the steps of (i) isolating a single-stranded polynucleotide from a viral vector or plasmid vector which has at least one starting polynucleotide insert; and (ii) conducting a polymerization process on the single-stranded polynucleotides of step (i) using random primers.

Step 2: The second step of the inventive method may comprise the steps of (i) conducting at least one cycle wherein the primers are extended to the end of the unidirectional single-stranded DNA fragments used as templates; (ii) conducting at least one subsequent cycle wherein each of the resulting extended polynucleotides of step (i) is further extended to the end of an unidirectional single-stranded DNA fragment other than the unidirectional single-stranded DNA fragment used in step (i) by means of template switching; and (iii) repeating step (ii) until recombinant polynucleotides of desired length are obtained.

Specifically, the unidirectional single-stranded polynucleotide fragments of various lengths prepared in Step 1 are mixed together, a specific oligonucleotide having a nucleotide sequence complementary to the single-stranded polynucleotide fragments are added thereto, and a polymerase chain reaction is carried out under a proper stringency. Then, the specific oligonucleotide serves as a primer of polymerase chain reaction and is

elongated gradually at one direction(5'→3') in each turn of reactions, whereby the recombination reaction occurs. The synthesized polynucleotides are separated into single strands by denaturation process and re-annealed. At this time, the synthesized polynucleotide may be annealed with other polynucleotide fragment containing a homologous sequence.

More specifically, a mixture of double-stranded polynucleotides can be denatured by heat and consequent polymerase chain reaction consists of the following three steps. First, double-stranded template DNA is treated at 90 to 98℃ for 10 sec to 5 min in order to separate into single-strands(denaturation). Thereafter, by lowering the temperature, previously added primers are annealed with a complementary single-stranded template DNA(annealing). This step is carried out at 40 to 72℃ for 10 sec to 2 min. Then, upon regulation of the temperature within a range of 70 to 78℃, four kinds of dNTPs(dATP, dGTP, dCTP, dTTP) in the reaction mixture begin to react and a DNA complementary to the template DNA is synthesized and elongated. The reaction time depends on the length of DNA being synthesized.

In case of producing various recombinant DNAs in such a manner from two or more polynucleotides having homologous nucleotide sequences, a polynucleotide may extend from an oligonucleotide primer, which is capable of hybridizing with at least one of the starting polynucleotides, up to the 5' end of the unidirectional single-stranded DNA fragment used as a template in a cycle of synthesis; and the resulting polynucleotide may further extend to the end of other unidirectional single-stranded polynucleotide originating from other starting polynucleotide by template switching in the next cycle. At this time, a recombination boundary is formed between the oligonucleotides synthesized by employing as templates unidirectional single-stranded polynucleotides originating from different starting polynucleotides.

In Step 2 for the extension of polynucleotide, the unidirectional single-stranded polynucleotide fragments prepared in Step 1 are employed only as templates for generating the recombinant DNAs and, accordingly, the primers added at the begining are extended gradually to one direction(5'→3') using

them as templates through the repetitive PCR to result in generation of recombinant polynucleotides.

In Step 2, the DNA recombination is conducted by periodically repeating the steps of denaturation, annealing and extension at the presence of DNA polymerase for the desired period. The degree of recombination depends on the homology between the groups of single-stranded polynucleotides derived from different starting polynucleotides.

Step 3: By sufficiently repeating the PCR cycles of Step 2 and amplifying the resulting mutant recombinant polynucleotides by a normal PCR method, a recombinant double-stranded DNA library is prepared. The recombinant DNA library thus obtained may consist of various kinds of mutant double-stranded polynucleotides which contain in a molecule the identical and heterogenous regions as compared with corresponding regions of any one of the starting double-stranded polynucleotides. The nucleotide sequence of the recombinant DNA may be determined by a conventional method, e.g., Maxam-Gilbert's method(Maxam, A. M and Gilbert, W., *Mol. Biol.(Mosk)*, *20*: 581-638 (1986)), Dideoxy method(Messing, J. et al., *Nucleic Acids Res.*, *24*; 309-321(1981)), or a method using DNA fluorescence marker and automated DNA sequence analyzer.

The present invention further provides a method for constructing a recombinant DNA library for screening a desired gene using the recombinant DNAs obtained by the above method. Specially, it comprises the steps of inserting the mutant recombinant double-stranded DNA obtained in Step 3 into an appropriate expression vector, introducing the resulting expression vector into an expression cell to obtain a library containing a plurality of clones; screening a desired polynucleotide from the clones; and expressing a protein from the polynucleotide by a conventional method. Suitable expression methods include: producing and accumulating a gene product in cells; secreting a gene product from a cell and accumulating them in a medium; secreting a gene product into a periplasm; and the like methods. For

screening a desired gene product from a recombinant DNA library, the methods known in the art, e.g. immunochemical method, radiochemical method, a method employing surface expressing system, and gene chip screening method, may be employed alone or in combination. In preparing the recombinant DNA library, any expression vector that operates in a selected host cell may be employed, exemplary vectors including conventional vectors of phage, plasmid, phagemid, viral vector and artificial chromosome known in the art. The method for constructing the expression vector is well known in the art, e.g., in Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual,* 2nd ed., (1989) Cold Spring Harbor Laboratory Press, N.Y. A suitable host cell may be transformed with the resulting expression vector. The suitable host cells for expressing the recombinant DNA include a bacterium such as *E. coli*, *Bacillus subtilis* and *B. brevis*, etc.; an Actinomyces such as *Streptomyces lividans*; a yeast such as *Saccharomyces serevisiae*; a fungus such as *Aspergillus oryzae*, *A. nidulans* and *A. niger*; an animal cell such as COS-7, CHO, Vero and mouse L cells; an insect cell; and a plant cell.

The present invention provides a method for preparing various, random, mutant recombinant DNAs in a short period of time. Specifically, a library of mutant recombinant polynucleotides can be obtained by adding oligonucleotide primers to a mixture of unidirectional single-stranded DNA fragments derived from two or more of homologous nucleic acid sequences or polynucleotides; and conducting repetitive PCR to obtain the library of mutant recombinant polynucleotides, wherein random recombinations between the nucleotide sequences of the single-stranded oligonucleotide fragments are occurred.

The recombinant DNAs prepared by the inventive method may be genes encoding proteins, e.g., enzymes, antibodies, vaccines(antigens), hormones, growth factors, binding proteins and plasma proteins. For instance, the recombinant DNA may encode an enzyme, said enzyme being selected from the group consisting of hydrolase, lyase, transferase, oxidoreductase, ligase and isomerase. A preferred embodiment of the present invention

provides a method for constructing a recombinant DNA library by preparing a recombinant gene(recombinant DNA) having a random mutation between *Serratia marcescens* chitinase gene(SEQ ID NO: 1, designated "*m-chi*") and *S. liquefaciens* chitinase gene(SEQ ID NO: 2, designated "*l-chi*") and cloning the recombinant gene. About 10,000 clones were prepared by the inventive method and, among them, 10 clones were randomly selected to determine the nucleotide sequences thereof. Comparison of their nucleotide sequences with those of the two wild-type genes exhibited that one time of recombination is occurred between the two genes in recombinant clones 3, 4 and 10; two times of recombinations, in recombinant clones 1, 2, 7 and 8; three times of recombinations, in recombinant clones 6 and 9; and four times of recombinations, in reocmbinant clone 5. These results demonstrate that the inventive method is effective in constructing a recombinant DNA library having a random recombination between two or more kinds of polynucleotides.

The inventive method for constructing a recombinant DNA library has a wide applicability. This *in vitro* mutagenization method may be used in a laboratory as means for biochemical studies. Since it allows to understand the mechanism of a protein involving in the maintenance and regulation of life in a molecular level, it may be used as means for producing and screening a proten such as an enzyme, antibody, vaccine(antigen), hormone, adsorption protein or plasma protein, thereby inducing the change of substrate specificity, change of reaction specificity, increase of activity, change of antigenicity, change of safety of a protein. Therefore, it is ultimately applied to various industrial fields for the development of a medicine, improvement and enhancement of food quality, improvement of energy conversion rate, breeding and quality improvement in livestock and fishery, development and production of novel chemical product, etc.(Chartrain M. et al., *Curr. Opin. In Biotech.*, *11*: 209-214 (2000); Miyazaki K. et al., *J. Mol. Biol.*, *297*: 1015-1026 (2000); Giver, L. and Arnold, F. H., *Curr. Opin. Chem. Biol.*, *2*: 335-338 (1998); Kumamaru, T. et al., *Nat. Biotechnol.*, *16*: 663-666 (1998); and Patten, P. A., *Curr. Opin. Biotechnol.*, *8*: 724-733 (1997)).

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usage and conditions.

Example 1 : Generating unidirectional single-stranded polynucleotide fragments

A pool of unidirectional single-stranded polynucleotide fragments having random length was prepared from a pair of double-stranded polynucleotides which have regions of similarity with each other as follows:

1-1) Preparation of unidirectional single-stranded DNA fragments by Reverse Transcription

In an embodiment of the present invention, genes encoding chitinase of *Serratia marcescens* and *Serratia liquefaciens* (hereinafter, referred to as "*m-chi*" and "*l-chi*", respectively) were chosen as starting polynucleotides to be reassembled, the nucleotide sequences of which are described in Fig. 2.

*Hin*dIII/*Xba* I fragments containing *m-chi* and *l-chi* genes, respectively, were cloned into pUC19, resulting in pUC19-*m-chi* and pUC19-*l-chi*. These plasmids were treated with *Nde* I , gap-filled with Klenow and then digested with *Hin*dIII. The resulting DNA inserts of about 2-kb were ligated to the *Hin*dIII/*Eco*RV backbone of pBluescriptII KS (Stratagene) to give 5-kb recombinant plasmids. The resulting plasmids, pBSK-*m-chi* and pBSK-*l-chi,* were then linearized with *Spe* I .

200 ng of the linearized plasmids was added to transcription buffer solution [40 mM Tris-HCl(pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT] supplemented with 0.5 mM each rNTP, 40 units of RNasin and 17 units of T3 RNA polymerase up to the total volume of 20$\mu\ell$

and incubated at 37℃ for 1 hour. The RNA transcripts of the *m-chi* and *l-chi* genes obtained by the above *in vitro* transcription were analyzed by electrophoresis on 1% agarose gel. The bands of 5-kb plasmid and RNA transcripts are detected in lanes 2 and 3 of Fig. 3 (a). Purified with RNAeasy column(Qiagen), 200 ng of each RNA transcribed from the two chitinase genes was mixed. The RNA mixture was added to the reaction buffer[10 mM Tris-HCl(pH 8.3), 15 mM KCl, 0.6 mM $MgCl_2$, 0.2 mM DTT] supplemented with 6 μg of random hexamer (Genotech, Inc.), 0.2 mM each dNTP, 40 units of RNasin and 50 units of M-MLV reverse transcriptase to the total volume of 50 μℓ and reverse transcription was performed at 37℃ for 1 hour. After the reverse transcription, the RNA templates were removed by incubating the reaction mixture with 20 ng of RNase I at 37℃ for 1 hour.

Since the random hexamer can be hybridized with the template RNA at all the location thereof by chance, nucleotide extension from the random hexamer generates unidirectional single-stranded DNA fragments with random length.

The products of reverse transcription were electrophorezed on 1% agarose gel (lane 2, Fig. 3 (b)) and the single-stranded DNA fragments were cut and purified using a Geneclean kit (Bio 101).

<u>1-2) Preparation of unidirectional single-strand DNA fragments with serial 5' deletions</u>

This method is based on two useful features of exonuclease III: (i) processive digestion at a very uniform rate and (ii) failure to initiate digestion at DNA ends with 4-base 3'-protrusions (Henikoff, S., *Gene* 28, 351-359, 1984).

Plasmid pGEM-T (Promega) having *m-chi* gene of 30μg was linearized with a pair of restriction enzymes, *Sph* I and *Nco* I, wherein *Sph* I produces 4-base 3'-protrusions resistant to the exonuclease III digestion while *Nco* I generates 4-base 5'-overhanging ends. As for *l-chi* gene, the above process was conducted as same. The linearized polynucleotides dissolved in exonuclease III reaction buffer[66 mM Tris-HCl (pH 8.0), 0.66

mM $MgCl_2$] up to the volume of 60$\mu\ell$ were digested with 2 units of exonuclease Ⅲ. 2.5$\mu\ell$ of aliquot was then removed at intervals of twenty seconds, and the enzyme reaction was terminated. The resulting aliquot was mixed with 7.5$\mu\ell$ of S1 nuclease mix [S1 nuclease reaction buffer(300 mM potassium acetate, pH 4.6, 2.5 M NaCl, 10 mM $ZnSO_4$, 50% glycerol) plus 50 units of S1 nuclease] and then placed at room temperature for 15 minutes.

After the S1 nuclease was inactivated by S1 stop solution[300 mM Tris base, 50 mM EDTA], polymerization was performed at 37℃ for 30 min by adding Klenow and then the products were cleaved with *Sac* I. The resulting double-stranded DNA fragments having random deletions sequentially were analyzed by electrophoresis on 1% agarose gel. The DNA fragments were extracted from the gel and reacted with 2 units of exonuclease Ⅲ for 1 hour to produce a set of single-stranded DNA fragments having unidirectional deletions thereon.

<u>1-3) Preparation of unidirectional single-strand DNA fragments using single-stranded DNA as a template</u>

Plasmid pGEM-T (Promega) having each of *m-chi and l-chi* genes of 5 μg was linearized with a pair of restriction enzymes, *Sph* I and *Nco* I. The linearized polynucleotides dissolved in exonuclease Ⅲ reaction buffer[66 mM Tris-HCl (pH 8.0), 0.66 mM $MgCl_2$] up to the volume of 60$\mu\ell$ were digested with 2 units of exonuclease Ⅲ at 37℃ for 30 min.

The resulting linearized single-stranded polynucleotides were used as templates to generate the single-stranded DNA fragments in a polymerization mix[10 units of Klenow, 6μg of random hexamers, 0.1 mM each dNTP, 10 mM Tris- HCl(pH 7.5), 5 mM $MgCl_2$, 7.5 mM DTT] at 37℃.

The resulting unidirectional single-stranded DNA fragments were analyzed by electrophoresis on 1% agarose gel and subsequently purified using a Geneclean kit(Bio 101).

Example 2 : Reassembly of polynucleotides by polymerase chain reaction using the unidirectional single-stranded DNA fragment as a template

The unidirectional single-stranded DNA fragments obtained above served as templates for polymerase chain reaction. A reaction mixture contained 20 ng of the single-stranded DNA fragments, 0.2 mM each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl(pH 8.8), 0.1% Triton X-100, 2 units of Vent DNA polymerase (New England BioLabs) and 25 pmole of a primer in a total volume of 50$\mu\ell$, wherein the primer being an oligonucleotide(SEQ ID NO: 24) having the nucleotide sequence identical to those at the 5' termini of *m-chi* and *l-chi* genes. PCR was carried out on an MJ Research thermal cycler (PTC-100) at 94℃ for 3 min; 94℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds (30 cycles); and 72℃, 5 min. For the amplification of a full-length DNA, secondary PCR was carried out on the above PCR products using 25 pmole of a 3'-specific oligonucleotide (SEQ ID NO: 25) as a primer. PCR was carried out on an MJ Research thermal cycler (PTC-100) at 94℃ for 3 min; 94℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds (30 cycles); and 72℃, 5 min. The resulting PCR products of about 1.7 kb were analyzed by 1% agarose gel electrophoresis (lane 2, Fig. 3 (c)).

Example 3 : Sequencing and screening

The PCR products of example 2 were extracted from the gel by a Geneclean kit(Bio 101), digested with *Hin*dIII and *Xba* I, and ligated to the *Hin*dIII/*Xba* I backbone of pBluescriptII KS. The resulting recombinant plasmid was transformed to *E. coli* JM83 and transformants were selected on LB-agar plates supplemented with 100μg/ml ampicillin. Plasmid DNA was isolated from the randomly chosen 14 colonies by Qiagen Spin Miniprep kit(Qiagen) and digested with restriction enzymes, *Not* I, *Pst* I and *Hinc* II.

Fig. 4 shows various sizes of DNA resolved by usual electrophoresis on 1% agarose gel. The band patterns of DNA fragments of *l-chi* gene cleaved with the same three restriction enzymes are shown in lane 5, those of *m-chi* gene in lanes 8 and 13. The remaining lanes represent patterns of random recombinant DNA reassembled from *m-chi* and *l-chi* genes, the

patterns different from those of wild type DNA fragments. These results show that at least 11 clones of the randomly selected 14 clones contain recombinant DNA reassembled from a pair of the wild-type DNA.

To identify the resulting recombinant DNA, *Hin*dIII/*Xba* I fragment of the 10 plasmids was sequenced using the ABI PRISM Dye terminator Cycle Sequencing Kit (PE Biosystems) and the sequences were compared with those of the wild-type *m-chi* and *l-chi* genes, alignments of which were shown in Fig. 5 and further depicted in the schematic diagram of Fig. 6.

As shown in Fig. 6, recombination between the two wild-type genes took place once as for the recombinant DNA clones 3, 4 and 10; twice as for clones 1, 2, 7 and 8; three times as for clones 6 and 9; and four times as for clone 5. These results suggest that the method of the present invention using unidirectional single-stranded polynucleotides can efficiently generate a random recombinant DNA library from two or more kinds of starting polynucleotides.

To screening a recombinant polynucleotide encoding a chitinase which has specific activity higher than that of wild-type enzyme, the colonies were transferred by replica-plating method to LB-agar plates containing 100μg/ml ampicillin and 0.5% swollen chitin, and incubated at 37℃ overnight until clear plaques were developed. About 800 colonies were screened according to the degree of their clearance. Fig. 7 shows the variance of the sizes of clear zones formed by the colonies expressing the recombinant chitinase depending on their chitin decomposing activities different from each other. A chitinase produced by a colony forming a clear zone larger than wild type was designated R-24 chitinase. Plasmid DNA was extracted from the clone by Qiaprep Spin Miniprep method(Qiagen) and the nucleotide sequence of R-24 chininase gene was analyzed. Fig. 8 compares the nucleotide sequence of R-24 chininase gene (SEQ ID NOs: 13) with those of two wild-type genes, i.e., *l-chi* gene(SEQ ID NO: 1) and *m-chi* gene(SEQ ID NO: 2). Fig. 9 is a schematic diagram showing the constitution of R-24 chininase gene in comparison with the two wild-type genes. From Fig. 9, it can be seen that R-24 chitinase gene was produced by four times of recmbinations between the

two wild-type genes.

Table 1 shows the comparison of the specific activity of R-24 chitinase with those of the two wild-type chitinases.

Table 1: Specific activities of the wild-type chitinases and recombinant R-24 chitinase

Chitinase	Specific activity (U/mg)
Serratia marcescens chitinase	150.6
Serratia liquefaciens chitinase	201.3
R-24 chitinase	227.2

As can be seen from Table 1, specific activity of R-24 chitinase is higher than *Serratia marcescens* chitinase and *Serratia liquefaciens* chitinase by factors of 1.5 and 1.1, respectively.

Example 4 : Directed evolution of a chitosanase for thermostability

4-1) Preparation of mutant chitosanases by error-prone PCR

About 0.5-kb DNA fragment obtained by *Eco*RV/*Sal* I double digestion of pBR322 was inserted into *Eco*RV/*Sal* I digestion site of pBluescript II SK. The resulting vector construct was then cut with *Xba* I and *Eco*R I, and ligated to about 1.4-kb chitosanase gene obtained by digesting *Bacillus sp.* (KCTC 0377BP) with same restriction enzymes, resulting in a recombinant vector construct, pBSK-csn-322, containing chitosanase gene.

The pBSK-csn-322 was used as a template for error-prone polymerase chain reaction. Each 50 pmole of primers csn-*Xba* I (SEQ ID NO: 26) and csn-c1(SEQ ID NO: 27) was used for an error-prone PCR reaction which was performed in 100$\mu\ell$ of PCR mix comprising 10 mM Tris-HCl(pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP 1 mM dCTP, 1 mM

dTTP, 0.15 mM $MnCl_2$, 10 ng of template DNA and 5 units of Taq polymerase using an MJ Research Thermal cycler (PTC-200). The PCR conditions were as follows: 94℃ for 3 min; 94℃ for 30 seconds, 55℃ for 30 seconds and 72℃ for 30 seconds (30 cycles); and followed by 72℃, 5 min.

The resulting 1.4-kb DNA fragment was digested with *Xba* I and *Eco*R I, then ligated to *Xba* I /*Eco*R I backbone of pBSK-csn-322. The resulting recombinant plasmid was transformed to *E. coli* JM83 and positive transformants were selected by culturing them on LB-agar plates supplemented with 100μg/ml ampicillin at 37℃ for 18 hours. The colonies formed on the plates were replica-plated onto a fresh plates and incubated at 37℃ for 20 hours. The petri dish containing the colonies was heated on a water bath at 70℃ for 15 minutes, and then 50 mM Na-acetate buffer solution containing 0.1% chitosan and 1% agarose was poured onto the LB-agar plates. After the plates was placed at 37℃ for 24 hours, colonies still having chitosanase activity to produce clear plaques were selected using 0.2 % Congo Red. As a result of aforementioned process, 9 positive clones having improved thermal stability were isolated out of about 12,000 clones. Plasmid DNAs were extracted from the clones by Qiaprep Spin Miniprep method(Qiagen) and the nucleotide sequences of chitosanase genes therein were analyzed. Table 2 shows the amino acid substitution sites of thermostable mutant chitosanases produced by error-prone PCR in comparison with the wild-type chitosanase.

Table 2: Amino acid substitution sites of thermostable mutant chitosanases produced by error-prone PCR

Mutant chitosanase	Amino acid substitution sites
d10-68	D305G
e3-97	E308G
e4-12	I389M
e15-20	T131I, N368D
e18-5	S24P, T277A, N368D
e22-23	K172E, S376P
e26-27	Q159R
e26-98	E107D, Q442R
e30-97	S376P, Y451C

<u>4-2) Construction of the first recombinant DNA library and screening</u>

DNA reassembly process according to the present invention was carried out using the 9 mutant chitosanase genes selected in 4-1) above as starting polynucleotides.

The plasmids extracted from the 9 clones were mixed in each quantity of 500 ng and then the linearized DNA fragments of about 4.9-kb in size were obtained by *Xho* I digestion. The linearized fragments of 200 ng were transcribed in 20$\mu\ell$ of transcription buffer solution[40 mM Tris-HCl, pH 7.8, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT] containing 0.5 mM each rNTPs, 40 units of RNasin and 17 unist of T3 RNA polymerase at 37℃ for 1 hour. The resulting RNA transcripts of the mutant genes for chitosanase were purified in RNAeasy column(Qiagen).

Reverse Transcription was conducted on 200 ng of the RNA in 50$\mu\ell$ of reaction solution[10 mM Tris-HCl, pH 8.3, 15 mM KCl, 0.6 mM $MgCl_2$, 0.2 mM DTT] containing 6μg of random hexamer, 0.2 mM each dNTPs, 40

units of RNasin and 50 units of M-MLV reverse transcriptase at 37℃ for 1 hour. The template RNA was then removed by RNase I at 37℃ for 1 hour. Through the reverse transcription process, unidirectional single-stranded DNAs of random size were synthesized from the random hexamer annealed with the template RNA. The resulting single-stranded DNA was analyzed by 1% agarose gel electrophoresis and extracted from the gel using a Geneclean kit(Bio 101).

The unidirectional single-stranded DNA fragments obtained above served as templates for polymerase chain reaction. A reaction mixture contained 10 ng of single-stranded DNA fragments, 0.2 mM each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl(pH 8.8), 0.1% Triton X-100, 2 units of Vent DNA polymerase (New England BioLabs) and 25 pmole of csn-*Xba* I primer(SEQ ID NO: 26) in a total volume of 50$\mu\ell$. PCR was carried out on an MJ Research thermal cycler (PTC-100) at 94℃ for 3 min; 94℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds (30 cycles); and 72℃, 5 min. The full-length DNA of about 1.4-kb in size was then amplified by PCR using 25 pmole of csn-c1 primer(SEQ ID NO: 27) under the same conditions as described above. The resulting 1.4-kb DNA was digested with *Xba* I and *Eco*R I and then ligated to the *Xba* I /*Eco*R I backbone of pBSK-csn-322. The resulting plasmid was transformed to *E. coli* KM83 and positive transformants were selected on LB-agar plates supplemented with 100μg/ml ampicillin at 37℃ for 20 hours. Grown colonies were transferred onto fresh plates by replica-plating method and incubated at 37℃ for 20 hours. The plates were heated on water bath at 75℃ for 20 minutes, and then 50 mM Na-acetate solution containing 0.1% chitosan and 1% agarose was added onto the LB-agar plates. After incubated at 37℃ overnight, colonies still having chitosanase activity resulting in clear plaque around them notwithstanding the heat treatment were selected on 0.2% Congo Red.

Through the aforementioned process, 23 clones having improvement in heat resistance compared to the 8 clones obtained by error-prone PCR were selected out of about 12,000 clones.

4-3) Construction of secondary recombinant DNA library and screening

Secondary recombinant DNA library was constructed with the 23 mutant chitosanase genes, which had been obtained by the screening of the first recombinant DNA library, through the same process as described in 4-2) above. After heated at 80℃ for 30 min, the resulting colonies of 16,000 or more were screened for mutant chitosanase having more improved thermal stability than the starting materials, 23 mutant chitosanases. Two mutants were selected and polypeptides encoded by them were designated as M-13 and M-20, respectively.

4-4) Determination of amino acid substitution sites and analysis for thermal stability of M-13 and M-20

From the colonies expressing the mutant chitosanases, M-13 and M-20, plasmid DNAs were extracted and the nucleotide sequences of the chitosanase genes were analyzed. Fig. 10 represents the comparison between the sequences of wild-type chitosanase and the genes encoding M-13 and M-20, respectively. Further, deduced amino acid sequences of the wild-type chitosanase and the thermostable M-13 and M-20 mutants were analyzed and the amino acid substitution sites of the mutant chitosanase different from those of the wild-type chitosanase were presented in Table 3.

Table 3: Amino acid substitution sites of thermostable mutant chitosanases produced by the inventive method

Mutant chitosanase	Amino acid substitution sites
M-13 chitosanase	N60Y, E107D, Q159R, N228T, D305G, E308G, N368D, S376P, F384L, I389M, D435G
M-20 chitosanase	S24P, E107D, Q159R, N286D, D305G, E308G, N357D, N368D, N371D

As can be seen from Table 3, when compared with the substitution sites present in the mutants prepared by the error-prone PCR as shown in Table 2, it

was exhibited that the substitution sites present in seven mutants, i.e., E107D, Q159R, D305G, E308G, N368D, S376P and I389M, were accumulated in M-13 chitosanase; and the substitution sites present in six mutants, i.e., S24P, E107D, Q159R, D305G, E308G and N368D, in M-13 chitosanase, by the recombination. This result demonstrates that the method of the present invention is useful for the efficient production of recombinant polynucleotides. On the other hand, it can be seen that in addition to the substitution sites resulted from the recombination between the parent mutants, new 4 and 3 mutation sites were introduced into M-13 and M-20 mutant chitosanases, respectively, during the process of the inventive method.

In order to determine the thermal stabilities of the mutant chitosanases, the wild-type chitosanase, M-13 mutant and M-20 mutant were treated at 60℃ and the remaining activities according to time were determined. Fig. 11 shows the differences in the thermal stabilities of the wild-type chitosanase, M-13 mutant and M-20 mutant. In Fig. 11, half-lives($T_{1/2}$) of the enzymes, which means that the activity thereof decreases by 50% as compared to the initial activity, are 5.1 min for the wild-type, 6.9 hours for M-13 mutant and 11.6 hours for M-20 mutant. This result shows that the thermal stabilities at 60℃ of M-13 and M-20 mutants increased by 81 and 136 folds, respectively, than the wild-type chitosanase.

As can be appreciated from the disclosure and the examples above, the method of the present invention can be used for *in vitro* recombination of homologous polynucleotides and the directed molecular evolution of proteins for desired properties. It is also contemplated that the method of the present invention has advantages over the conventional methods in that random diversity of the polynucleotides is achieved in a short time.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

<u>What is claimed is</u>:

1. A method for producing recombinant polynucleotides comprising the steps of:

(a) generating a pool of unidirectional single-stranded polynucleotide fragments randomized in length from at least one starting polynucleotide to be reassembled, which have regions of similarity with each other;

(b) conducting a polymerization process comprising multi-cyclic extension reactions, wherein the unidirectional single-stranded polynucleotide fragments prepared by step (a) serve as templates sequentially and specific oligonucleotides are added to the reaction mixture as primers, the primers being extended by means of template switching to produce at least one recombinant polynucleotide, and the resulting recombinant polynucleotides being different from the starting polynucleotides in nucleotide sequence; and

(c) conducting a polymerase chain reaction using at least one specific primer to amplify the recombinant polynucleotides prepared by step (b).

2. The method of claim 1, wherein step (a) comprises:

(i) conducting a transcription process to produce RNA from at least one starting polynucleotide; and

(ii) conducting a reverse transcription process, wherein random primers are used as primers and the RNA transcript of step (i) as a template.

3. The method of claim 1, wherein step (a) comprises:

(i) generating a 3'-overhang on one side of the starting double-stranded polynucleotide by digesting with at least one restriction enzyme;

(ii) producing a pool of double-stranded polynucleotides having unidirectional sequential deletion by treating the reaction mixture of step (i) with exonuclease III followed by removing aliquots of the reaction mixture at

a chosen time interval and further blocking the activity of the exonuclease Ⅲ;

(iii) treating the resulting double-stranded polynucleotides having a 5'-overhang with an S1 nuclease and a DNA polymerase to form a blunt end thereof;

(iv) generating a new 3'-overhang to the same side which has 3'-overhang in step (i); and

(v) treating the polynucleotides of step (iv) with exonuclease Ⅲ to generate single-stranded polynucleotide fragments.

4. The method of claim 1, wherein step (a) comprises:

(i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme;

(ii) treating the polynucleotides of step (i) with exonuclease Ⅲ to generate single-stranded polynucleotides; and

(iii) conducting a polymerization process on the single-stranded polynucleotides of step (ii) using random primers.

5. The method of claim 1, wherein step (a) comprises:

(i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme;

(ii) treating the polynucleotides of step (i) with exonuclease Ⅲ to generate single-stranded polynucleotides; and

(iii) producing a pool of single-stranded polynucleotides having unidirectional sequential deletion by treating the single-stranded polynucleotides of step (ii) with a single-strand specific 5'→3' exonuclease followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease.

6. The method of claim 1, wherein step (a) comprises:

(i) conducting a polymerase chain reaction on the starting double-stranded polynucleotides using only one kind of oligonucleotide among forward and reverse primers;

(ii) isolating the resulting single-stranded polynucleotides from the starting double-stranded polynucleotides; and

(iii) conducting a polymerization process on the single-stranded polynucleotides of step (ii) using random primers.

7. The method of claim 1, wherein step (a) comprises:

(i) conducting a polymerase chain reaction on the starting double-stranded polynucleotides using only one kind of oligonucleotide among forward and reverse primers;

(ii) isolating the resulting single-stranded polynucleotides from the starting double-stranded polynucleotides; and

(iii) producing a pool of single-stranded polynucleotides having unidirectional sequential deletion by treating the single-stranded polynucleotides of step (ii) with a single-strand specific 5'→3' exonuclease followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease.

8. The method of claim 1, wherein step (a) comprises:

(i) isolating a single-stranded polynucleotide from a viral vector or plasmid vector which has at least one starting polynucleotide insert; and

(ii) conducting a polymerization process on the single-stranded polynucleotides of step (i) using random primers.

9. The method of claim 1, wherein step (b) comprises the steps of:

(i) conducting at least one cycle wherein the primers are extended to the end of the unidirectional single-stranded DNA fragments used as templates;

(ii) conducting at least one subsequent cycle wherein each of the resulting extended polynucleotides of step (i) is further extended to the end of an unidirectional single-stranded DNA fragment other than the unidirectional single-stranded DNA fragment used in step (i) by means of template switching; and

(iii) repeating step (ii) until recombinant polynucleotides of desired length are obtained.

10. The method of claim 1, wherein the specific oligonucleotides of step (b) have specific nucleotide sequences which is capable of hybridizing with at least one starting polynucleotide.

11. The method of claim 1, wherein the starting polynucleotide is a gene encoding any one of proteins selected from the group consisting of enzymes, antibodies, antigens, binding proteins, hormones, growth factors and plasma proteins, or a part thereof.

12. The method of claim 11, wherein the enzyme is selected from the group consisting of hydrolase, lyase, transferase, oxidoreductase, ligase and isomerase.

13. The method of claim 1, wherein the starting polynucleotide is a wild type DNA or a mutant type DNA obtained therefrom.

14. A method for constructing a recombinant DNA library, comprising the steps of inserting the recombinant polynucleotide prepared by the method of any one of claims 1 to 10 into a vector; and transforming an expression cell with said vector containing the recombinant polynucleotide to obtain a

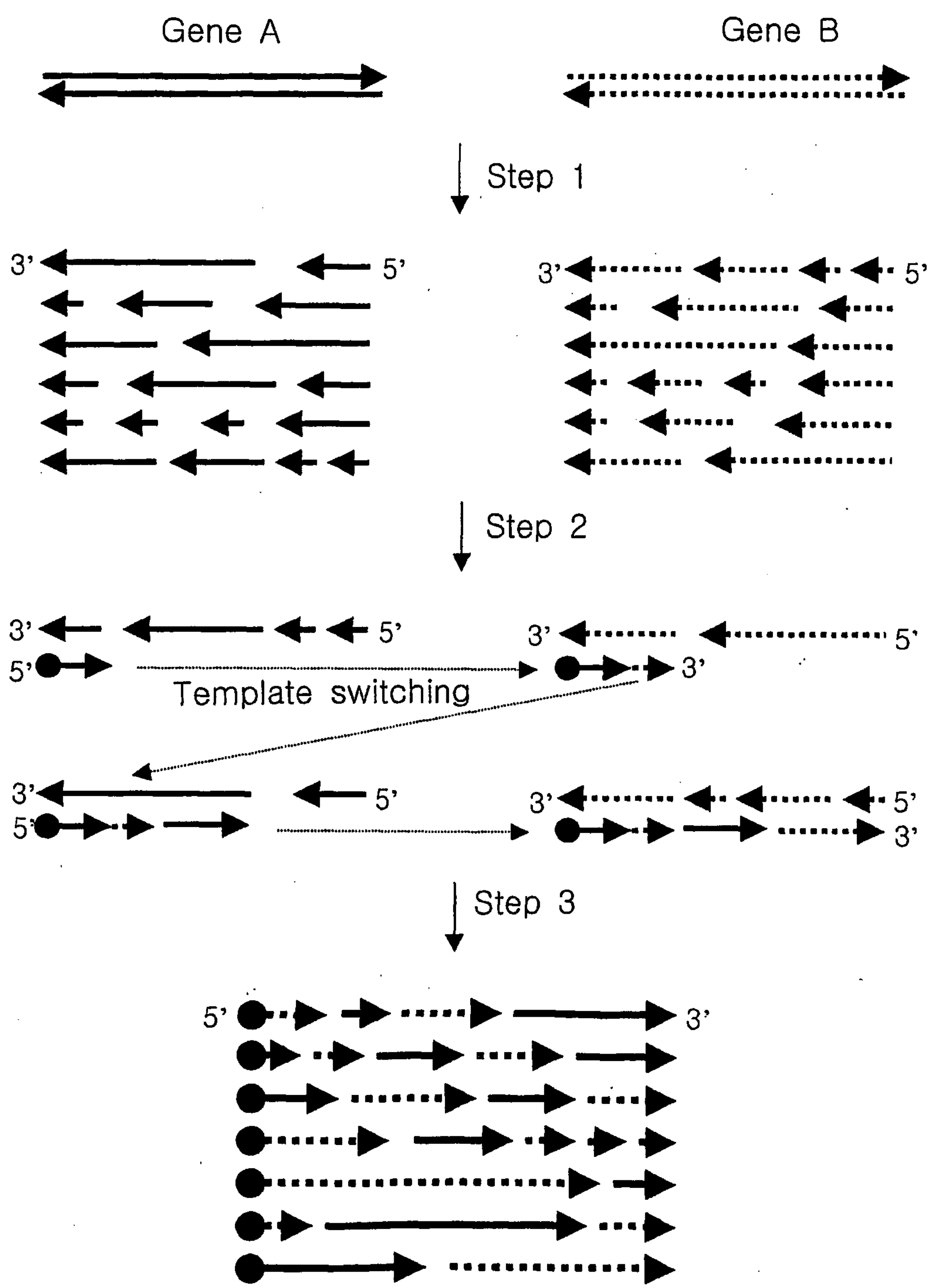
plurality of mutant clones.

15. The method of claim 14, wherein the vector is selected from the group consisting of a phage, a plasmid, a phagemid, a viral vector and an artificial chromosome.

16. The method of claim 14, wherein the expression cell is selected from the group consisting of bacteria, fungi, plant cells, animal cells and insect cells.

17. A method for evolving a polynucleotide toward a desired property which comprises screening recombinant polynucleotides having a desired functional properties from the recombinant DNA library constructed by the method of claim 14.

FIG. 1



Gene A
Gene B
Step 1
3'
5'
3'
5'
Step 2
3'
5'
3'
5'
5'
3'
Template switching
3'
5'
3'
5'
5'
3'
5'
3'
Step 3
5'
3'

FIG. 2

```
l-chi   ATGCGCAAAT TTAATAAACC GCTGTTGGCG tTGcTGATCG GCAGCACGCT    50
m-chi   ATGCGCAAAT TTAATAAACC GCTGTTGGCG cTGtTGATCG GCAGCACGCT    50

l-chi   GTGcTCtGCG GCGCAGGCCG CtGCaCCGGG CAAaCCtACg tTgGCCTGGG   100
m-chi   GTGtTCcGCG GCGCAGGCCG CcGCgCCGGG CAAgCCgACc aTcGCCTGGG   100

l-chi   GCAAtACCAA aTTCGCCATt GTcGAAGTcG AtCAaGCGGC gACgGCTTAT   150
m-chi   GCAAcACCAA gTTCGCCATc GTtGAAGTtG AcCAgGCGGC tACcGCTTAT   150

l-chi   AATAATcTGG TGAAaGTAAA AAgTGCCGCC GAcGTTTCtG TtTCaTGGAA   200
m-chi   AATAATtTGG TGAAgGTAAA AAaTGCCGCC GAtGTTTCcG TcTCcTGGAA   200

l-chi   TTTATGGAAT GGCGAtaCcG GtACcACGGC aAAagTaTTA TTAAATGGcA   250
m-chi   TTTATGGAAT GGCGAcgCgG GcACgACGGC cAAgaTtTTA TTAAATGGtA   250

l-chi   AAGAaGttTG GAGTGGTgCc TCAACCGGta gTTCgGGaAC cGCaAAcTTT   300
m-chi   AAGAgGcgTG GAGTGGTcCt TCAACCGGat cTTCcGGtAC gGCgAAtTTT   300

l-chi   AAggtgaATA AAGGCGGCCG TTATCAAATG CAGGTGGCgT TaTGCAAcGC   350
m-chi   AAaagtgATA AAGGCGGCCG TTATCAAATG CAGGTGGcaT TgTGCAAtGC   350

l-chi   CGACGGCTGt ACCGCCAGcG AtGCaACCGA AATTGTGGTG GCaGAtACCG   400
m-chi   CGACGGCTGc ACCGCCAGtG AcGCcACCGA AATTGTGGTG GCcGAcACCG   400

l-chi   ACGGtAGCCA TTTGGCaCCt TTaAAAGAaC CttTGtTGGA AAAGAATAAg   450
m-chi   ACGGcAGCCA TTTGGCgCCg TTgAAAGAgC CgcTGcTGGA AAAGAATAAa   450

l-chi   CCtTATAAAC AagACTCCGG CAAAGTGGTt GGcTCTTATT TCGTtGAaTG   500
m-chi   CCgTATAAAC AgaACTCCGG CAAAGTGGTc GGtTCTTATT TCGTcGAgTG   500

l-chi   GGGCGTTTAC GGcCGtAATT TCACCGTCGA tAAacTtCCG GCtCAgAACC   550
m-chi   GGGCGTTTAC GGgCGcAATT TCACCGTCGA cAAgaTcCCG GCgCAaAACC   550

l-chi   TGACgCACCT GCTGTACGGC TTTATCCCtA TCTGtGGCGG tgAcGGCATC   600
m-chi   TGACcCACCT GCTGTACGGC TTTATCCCgA TCTGcGGCGG caAtGGCATC   600

l-chi   AACGACAGCC TGAAAGAGAT cGAAGGCAGC TTCCAGGCGT TACAGCGtTC   650
m-chi   AACGACAGCC TGAAAGAGAT tGAAGGCAGC TTCCAGGCGT TACAGCGcTC   650

l-chi   CTGtCAGGGg CGtGAaGACT TtAAgGTaTC GaTCCACGAT CCGTTCGCtG   700
m-chi   CTGcCAGGGc CGcGAgGACT TcAAaGTcTC GgTCCACGAT CCGTTCGCcG   700

l-chi   CGCTGCAgAA AGgtCAGAAG GGCGTGACCG CCTGGGAcGA CCCCTACAAa   750
m-chi   CGCTGCAaAA AGcgCAGAAG GGCGTGACCG CCTGGGAtGA CCCCTACAAg   750

l-chi   GGCAACTTCG GCCAGtTGAT GGCGtTGAAa CAGGCGCgcC CgGACCTGAA   800
m-chi   GGCAACTTCG GCCAGcTGAT GGCGcTGAAg CAGGCGCatC CtGACCTGAA   800

l-chi   AATCCTGCCG TCGATCGGtG GCTGGACGtT aTCCGAtCCG TTCTTCTTtA   850
m-chi   AATCCTGCCG TCGATCGGcG GCTGGACGcT gTCCGAcCCG TTCTTCTTcA   850
```

FIG. 2 (continued)

l-chi	TGGGCGAtAA	GGTGAAGCGC	GATCGCTTCG	TCGGcTCGGT	GAAgGAGTTC	900
m-chi	TGGGCGAcAA	GGTGAAGCGC	GATCGCTTCG	TCGGtTCGGT	GAAaGAGTTC	900
l-chi	CTGCAaACCT	GGAAGTTCTT	tGAtGGCGTa	GATATCGACT	GGGAaTTCCC	950
m-chi	CTGCAgACCT	GGAAGTTCTT	cGAcGGCGTg	GATATCGACT	GGGAgTTCCC	950
l-chi	GGGCGGgcAg	GGtGCtAACC	CgAAaCTGGG	CAGtaCGCAg	GAtGGGGcAA	1000
m-chi	GGGCGGcaAa	GGcGCcAACC	CtAAcCTGGG	CAGccCGCAa	GAcGGGGaAA	1000
l-chi	CCTATGTGca	GCTGATGAAa	GAGCTGCGcG	CcATGCTGGA	TCAGCTtTCG	1050
m-chi	CCTATGTGtt	GCTGATGAAg	GAGCTGCGgG	CgATGCTGGA	TCAGCTgTCG	1050
l-chi	GCGGAAACgG	GCCGtAAGTA	TGAaCTGACC	TCtGCgATCA	GCGCCGGcAA	1100
m-chi	GCGGAAACcG	GCCGcAAGTA	TGAgCTGACC	TCcGCcATCA	GCGCCGGtAA	1100
l-chi	GGAtAAaATC	GAtAAGGTGG	aTTACAACac	cGCaCAaAAC	TCGATGGATC	1150
m-chi	GGAcAAgATC	GAcAAGGTGG	cTTACAACgt	tGCgCAgAAC	TCGATGGATC	1150
l-chi	ACATtTTCCT	GATGAGtTAC	GACTTCTATG	GgGCaTTCGA	TCTGAAaAAt	1200
m-chi	ACATcTTCCT	GATGAGcTAC	GACTTCTATG	GcGCcTTCGA	TCTGAAgAAc	1200
l-chi	CTGGGcCAcC	AGACtGCGCT	GAAaGCGCCG	GCCTGGAAaC	CGGAtACgGC	1250
m-chi	CTGGGgCAtC	AGACcGCGCT	GAAtGCGCCG	GCCTGGAAgC	CGGAcACcGC	1250
l-chi	gTAtACCACG	GTGAAtGGCG	TtAATGCaCT	GCTcaCGCAG	GGCGTgAAGC	1300
m-chi	tTAcACCACG	GTGAAcGGCG	TcAATGCgCT	GCTggCGCAG	GGCGTcAAGC	1300
l-chi	CGGGCAAaAT	CGTGGTgGGC	ACCGCCATGT	AcGGtCGCGG	tTGGACCGGG	1350
m-chi	CGGGCAAgAT	CGTGGTcGGC	ACCGCCATGT	AtGGcCGCGG	cTGGACCGGG	1350
l-chi	GTGAACGGtT	ACCAGAACAA	CATTCCGTTt	ACCGGcACCG	CCACTGGcCC	1400
m-chi	GTGAACGGcT	ACCAGAACAA	CATTCCGTTc	ACCGGtACCG	CCACTGGgCC	1400
l-chi	GGTgAAAGGC	ACCTGGGAaA	AtGGCATCGT	GGAtTACCGC	CAgATCGCCa	1450
m-chi	GGTtAAAGGC	ACCTGGGAgA	AcGGCATCGT	GGAcTACCGC	CAaATCGCCg	1450
l-chi	atgAGTTtAT	GAGCGGCGAa	TGGCAGTAcA	gCTACGAtGC	tACcGCtGAA	1500
m-chi	gccAGTTcAT	GAGCGGCGAg	TGGCAGTAtA	cCTACGAcGC	cACgGCgGAA	1500
l-chi	GCaCCcTAtG	TcTTCAAACC	TTCCACtGGC	GATCTGATCA	CCTTCGACGA	1550
m-chi	GCgCCtTAcG	TgTTCAAACC	TTCCACcGGC	GATCTGATCA	CCTTCGACGA	1550
l-chi	TGCgCGCTCG	GTGCAGGCgA	AgGGCAAaTA	tGTGCTGGAT	AAGCAGCTGG	1600
m-chi	TGCcCGCTCG	GTGCAGGCcA	AaGGCAAgTA	cGTGCTGGAT	AAGCAGCTGG	1600
l-chi	GCGGgtTGTT	CTCaTGGGAa	ATtGACGCcG	AcAACGGCGA	TATTCTgAAt	1650
m-chi	GCGGccTGTT	CTCcTGGGAg	ATcGACGCgG	AtAACGGCGA	TATTCTcAAc	1650
l-chi	AaCATGAACa	gCAGCCTGGG	CAACAGCGtC	GGtacgCctT	AA	1692
m-chi	AgCATGAACg	cCAGCCTGGG	CAACAGCGcC	GGcgttCaaT	AA	1692

FIG. 3

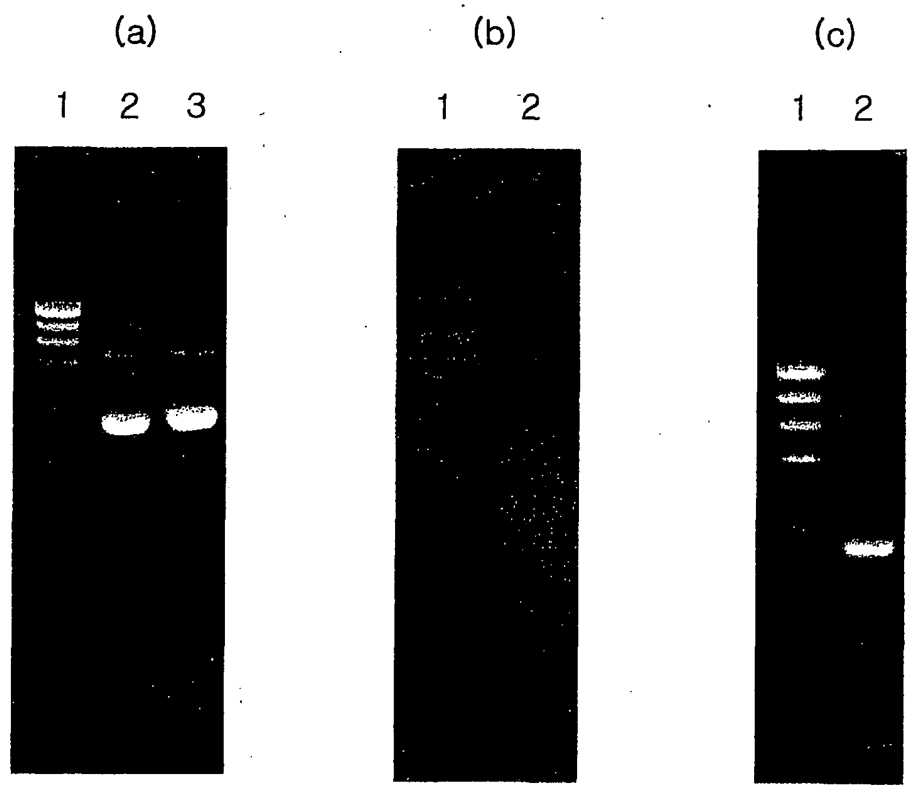


FIG. 4

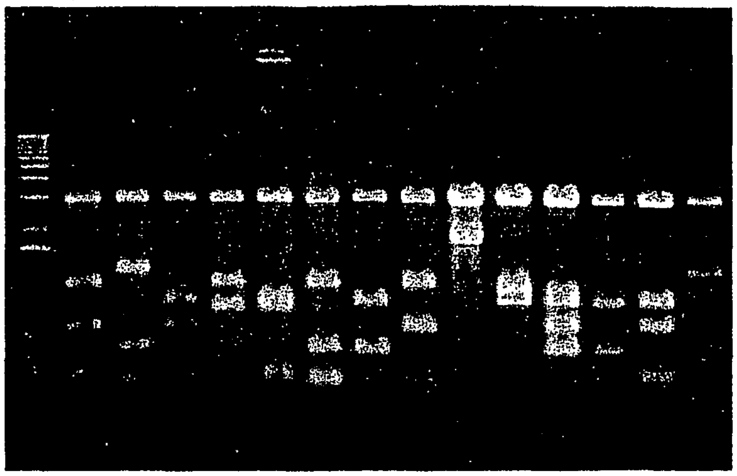


FIG. 5

```
l-chi    ATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCG 60
m-chi    ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
mut-1    ATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCG 60
mut-2    ATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCG 60
mut-3    ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
mut-4    ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
mut-5    ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
mut-6    ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
mut-7    ATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCG 60
mut-8    ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
mut-9    ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
mut-10   ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60

l-chi    GCGCAGGCCGCTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATT 120
m-chi    GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC 120
mut-1    GCGCAGGCCGCTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATT 120
mut-2    GCGCAGGCCGCTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATT 120
mut-3    GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC 120
mut-4    GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC 120
mut-5    GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC 120
mut-6    GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC 120
mut-7    GCGCAGGCCGCTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATT 120
mut-8    GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC 120
mut-9    GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC 120
mut-10   GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC 120

l-chi    GTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC 180
m-chi    GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC 180
mut-1    GTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC 180
mut-2    GTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC 180
mut-3    GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC 180
mut-4    GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC 180
mut-5    GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC 180
mut-6    GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC 180
mut-7    GTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC 180
mut-8    GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC 180
mut-9    GTTGAAGTTGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC 180
mut-10   GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC 180
```

FIG. 5 (continued)

```
l-chi    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
m-chi    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-1    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
mut-2    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
mut-3    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-4    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-5    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-6    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-7    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
mut-8    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-9    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
mut-10   GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240

l-chi    TTAAATGGCAAAGAAGTTTGGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
m-chi    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-1    TTAAATGGCAAAGAAGTTTGGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
mut-2    TTAAATGGCAAAGAAGTTTGGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
mut-3    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-4    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-5    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-6    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-7    TTAAATGGCAAAGAAGTTTGGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
mut-8    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-9    TTAAATGGCAAAGAAGTTTAGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
mut-10   TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300

l-chi    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
m-chi    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-1    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-2    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-3    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-4    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-5    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-6    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-7    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-8    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-9    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-10   AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
```

FIG. 5 (continued)

```
l-chi    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
m-chi    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-1    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-2    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-3    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-4    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-5    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-6    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-7    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-8    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-9    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-10   ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420

l-chi    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
m-chi    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-1    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTC 480
mut-2    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
mut-3    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-4    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-5    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-6    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
mut-7    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
mut-8    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-9    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
mut-10   TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480

l-chi    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
m-chi    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-1    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-2    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-3    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-4    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-5    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-6    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-7    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-8    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-9    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-10   GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
```

FIG. 5 (continued)

```
l-chi    GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
m-chi    GCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAATGGCATC 600
mut-1    GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-2    GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-3    GCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAATGGCATC 600
mut-4    GCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-5    GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-6    GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-7    GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-8    GCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAATGGCATC 600
mut-9    GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-10   GCGCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGTGATGGCATC 600

l-chi    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
m-chi    AACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGCCAGGGC 660
mut-1    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-2    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-3    AACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGCCAGGGC 660
mut-4    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-5    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-6    AACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-7    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-8    AACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGCCAGGGC 660
mut-9    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-10   AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGTCAGGGG 660

l-chi    CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
m-chi    CGCGAGGACTTCAAAGTCTCGGTCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
mut-1    CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-2    CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-3    CGCGAGGACTTCAAAGTCTCGGTCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
mut-4    CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-5    CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-6    CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-7    CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
mut-8    CGCGAGGACTTCAAAGTCTCGGTCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
mut-9    CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCCGAAAGGTCAGAAG 720
mut-10   CGCGAAGACTTCAAGGTATCGGTCCACGATCCGTTCGCCGCGCTGCAGAAAGGGCAGAAG 720
```

FIG. 5(continued)

```
l-chi    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
m-chi    GGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780
mut-1    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-2    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-3    GGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780
mut-4    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-5    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-6    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-7    GGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780
mut-8    GGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780
mut-9    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-10   GGCGTGACCGCCTGGGACGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780

l-chi    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
m-chi    CAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG 840
mut-1    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-2    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-3    CAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGTTATCCGATCCG 840
mut-4    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-5    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-6    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-7    CAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG 840
mut-8    CAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG 840
mut-9    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-10   CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840

l-chi    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
m-chi    TTCTTCTTCATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900
mut-1    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
mut-2    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAAGAGTTC 900
mut-3    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
mut-4    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
mut-5    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAAGAGTTC 900
mut-6    TTCTTCTTTATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900
mut-7    TTCTTCTTCATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900
mut-8    TTCTTCTTAATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900
mut-9    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
mut-10   TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
```

FIG. 5(continued)

```
l-chi     CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
m-chi     CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-1     CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
mut-2     CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-3     CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
mut-4     CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
mut-5     CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-6     CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-7     CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCCAA 960
mut-8     CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-9     CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
mut-10    CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960

l-chi     GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
m-chi     GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-1     GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
mut-2     GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-3     GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
mut-4     GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
mut-5     GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-6     GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-7     GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-8     GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-9     GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
mut-10    GGTGCTAACCCGAAACTGGGCAGTATGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020

l-chi     GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
m-chi     GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-1     GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
mut-2     GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-3     GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
mut-4     GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
mut-5     GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-6     GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-7     GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-8     GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-9     GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
mut-10    GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
```

FIG. 5 (continued)

```
l-chi    TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC 1140
m-chi    TCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAAC 1140
mut-1    TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC 1140
mut-2    TCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAAC 1140
mut-3    TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC 1140
mut-4    TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC 1140
mut-5    TCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAAC 1140
mut-6    TCCGCCATCAGCGCCGGTAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC 1140
mut-7    TCCGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC 1140
mut-8    TCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAAC 1140
mut-9    TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC 1140
mut-10   TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC 1140

l-chi    TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT 1200
m-chi    TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC 1200
mut-1    TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT 1200
mut-2    TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC 1200
mut-3    TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT 1200
mut-4    TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT 1200
mut-5    TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC 1200
mut-6    TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT 1200
mut-7    TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT 1200
mut-8    TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC 1200
mut-9    TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCCTTCGATCTGAAGAAC 1200
mut-10   TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT 1200

l-chi    CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG 1260
m-chi    CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG 1260
mut-1    CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG 1260
mut-2    CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG 1260
mut-3    CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG 1260
mut-4    CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG 1260
mut-5    CTGGGGCATCAGACCGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG 1260
mut-6    CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG 1260
mut-7    CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG 1260
mut-8    CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG 1260
mut-9    CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG 1260
mut-10   CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG 1260
```

FIG. 5 (continued)

l-chi	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
m-chi	GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAGATCGTGGTCGGC	1320
mut-1	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-2	GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAGATCGTGGTCGGC	1320
mut-3	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-4	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-5	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-6	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-7	GTGAATGGCGTTAATGCACTGCTCGCGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-8	GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAGATCGTGGTCGGC	1320
mut-9	GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-10	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320

l-chi	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
m-chi	ACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCGTTC	1380
mut-1	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-2	ACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCGTTC	1380
mut-3	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-4	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-5	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-6	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-7	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-8	ACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCGTTC	1380
mut-9	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-10	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380

l-chi	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
m-chi	ACCGGTACCGCCACTGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGC	1440
mut-1	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-2	ACCGGTACCGCCACTGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGC	1440
mut-3	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-4	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-5	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-6	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-7	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-8	ACCGGTACCGCCACTGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGC	1440
mut-9	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-10	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440

FIG. 5(continued)

l-chi	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
m-chi	CAAATCGCCGGCCAGTTCATGAGCGGCGAGTGGCAGTATACCTACGACGCCACGGCGGAA	1500
mut-1	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
mut-2	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
mut-3	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
mut-4	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
mut-5	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
mut-6	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
mut-7	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
mut-8	CAAATCGCCGGCCAGTTCATGAGCGGCGAGTGGCAGTATACCTACGACGCCACGGCGGAA	1500
mut-9	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500
mut-10	CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA	1500

l-chi	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560
m-chi	GCGCCTTACGTGTTCAAACCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGCTCG	1560
mut-1	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560
mut-2	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560
mut-3	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560
mut-4	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560
mut-5	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCCCGCTCG	1560
mut-5	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCCCGCTCG	1560
mut-6	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560
mut-7	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560
mut-8	GCGCCTTACGTGTTCAAACCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGCTCG	1560
mut-9	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560
mut-10	GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG	1560

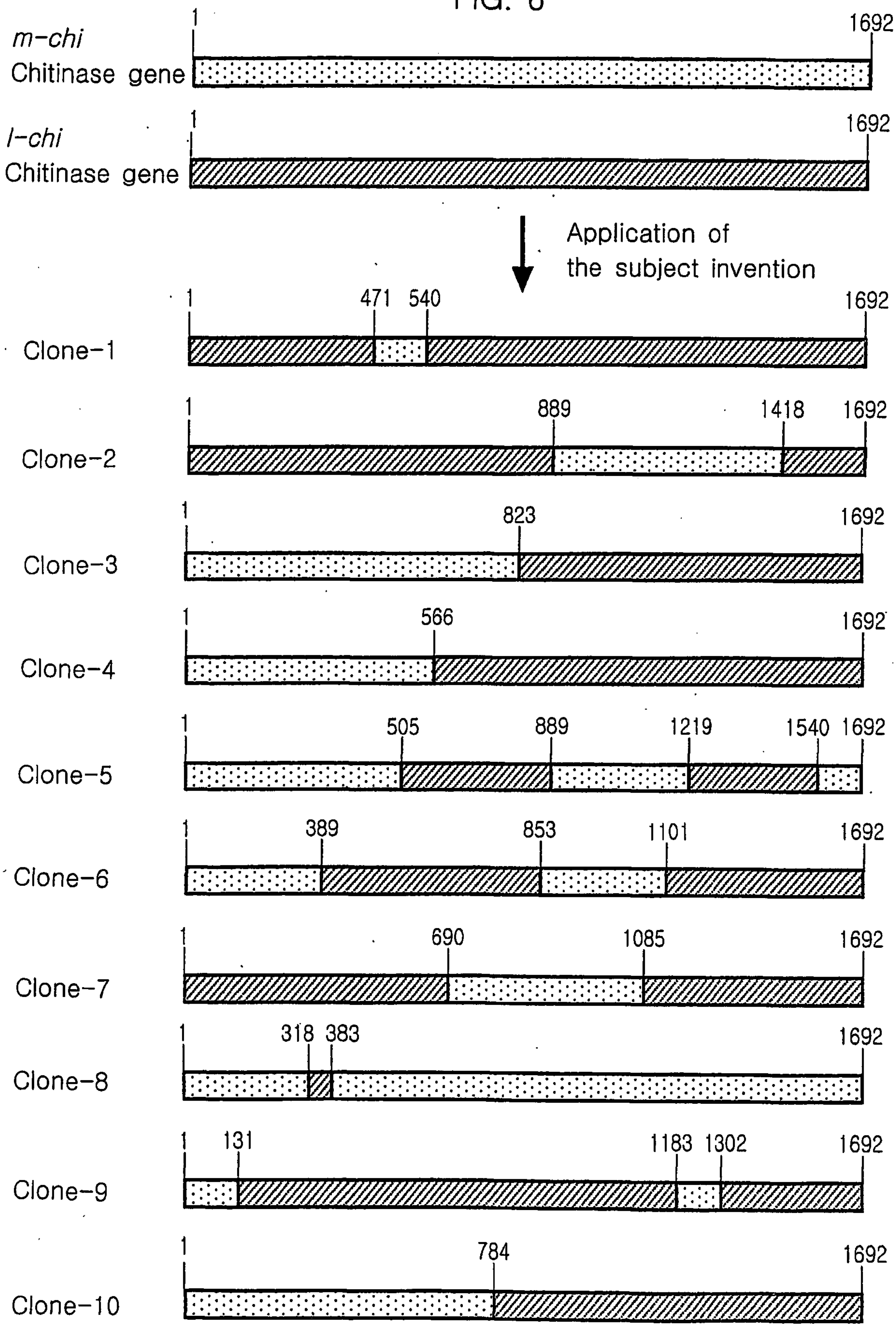
l-chi	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620
m-chi	GTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCCTGGGAG	1620
mut-1	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620
mut-2	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620
mut-3	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620
mut-4	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620
mut-5	GTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCCTGGGAG	1620
mut-6	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620
mut-7	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620
mut-8	GTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCCTGGGAG	1620
mut-9	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620
mut-10	GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA	1620

FIG. 5 (continued)

l-chi	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680
m-chi	ATCGACGCGGATAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAACAGCGCC	1680
mut-1	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680
mut-2	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680
mut-3	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680
mut-4	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680
mut-5	ATCGACGCGGATAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAACAGCGCC	1680
mut-6	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680
mut-7	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680
mut-8	ATCGACGCGGATAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAACAGCGCC	1680
mut-9	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680
mut-10	ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC	1680

l-chi	GGTACGCCTTAA	1692
m-chi	GGCGTTCAATAA	1692
mut-1	GGTACGCCTTAA	1692
mut-2	GGTACGCCTTAA	1692
mut-3	GGTACGCCTTAA	1692
mut-4	GGTACGCCTTAA	1692
mut-5	GGCGTTCAATAA	1692
mut-6	GGTACGCCTTAA	1692
mut-7	GGTACGCCTTAA	1692
mut-8	GGCGTTCAATAA	1692
mut-9	GGTACGCCTTAA	1692
mut-10	GGTACGCCTTAA	1692

FIG. 6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l-chi
Chitinase gene
1
1692
Application of
the subject invention
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1692
Clone-2
1
889
1418
1692
Clone-3
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823
1692
Clone-4
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505
889
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1540
1692
Clone-6
1
389
853
1101
1692
Clone-7
1
690
1085
1692
Clone-8
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1692
Clone-9
1
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1183
1302
1692
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1
784
1692

FIG. 7

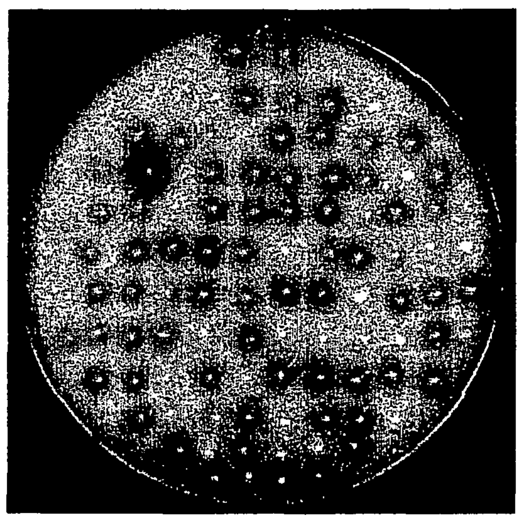

FIG. 8

l-chi	ATGCGCAAAT	TTAATAAACC	GCTGTTGGCG	TTGCTGATCG	GCAGCACGCT	50
m-chi	ATGCGCAAAT	TTAATAAACC	GCTGTTGGCG	CTGTTGATCG	GCAGCACGCT	50
R-24	ATGCGCAAAT	TTAATAAACC	GCTGTTGGCG	TTGCTGATCG	GCAGCACGCT	50
l-chi	GTGCTCTGCG	GCGCAGGCCG	CTGCACCGGG	CAAACCTACG	TTGGCCTGGG	100
m-chi	GTGTTCCGCG	GCGCAGGCCG	CCGCGCCGGG	CAAGCCGACC	ATCGCCTGGG	100
R-24	GTGCTCTGCG	GCGCAGGCCG	CTGCACCGGG	CAAACCTACG	TTGGCCTGGG	100
l-chi	GCAATACCAA	ATTCGCCATT	GTCGAAGTCG	ATCAAGCGGC	GACGGCTTAT	150
m-chi	GCAACACCAA	GTTCGCCATC	GTTGAAGTTG	ACCAGGCGGC	TACCGCTTAT	150
R-24	GCAATACCAA	ATTCGCCATT	GTCGAAGTCG	ATCAAGCGGC	GACGGCTTAT	150
l-chi	AATAATCTGG	TGAAAGTAAA	AAGTGCCGCC	GACGTTTCTG	TTTCATGGAA	200
m-chi	AATAATTTGG	TGAAGGTAAA	AAATGCCGCC	GATGTTTCCG	TCTCCTGGAA	200
R-24	AATAATCTGG	TGAAAGTAAA	AAGTGCCGCC	GACGTTTCTG	TTTCATGGAA	200
l-chi	TTTATGGAAT	GGCGATACCG	GTACCACGGC	AAAAGTATTA	TTAAATGGCA	250
m-chi	TTTATGGAAT	GGCGACGCGG	GCACGACGGC	CAAGATTTTA	TTAAATGGTA	250
R-24	TTTATGGAAT	GGCGATACCG	GTACCACGGC	AAAAGTATTA	TTAAATGGCA	250
l-chi	AAGAAGTTTG	GAGTGGTGCC	TCAACCGGTA	GTTCGGGAAC	CGCAAACTTT	300
m-chi	AAGAGGCGTG	GAGTGGTCCT	TCAACCGGAT	CTTCCGGTAC	GGCGAATTTT	300
R-24	AAGAAGTTTG	GAGTGGTGCC	TCAACCGGTA	GTTCGGGAAC	CGCAAACTTT	300
l-chi	AAGGTGAATA	AAGGCGGCCG	TTATCAAATG	CAGGTGGCGT	TATGCAACGC	350
m-chi	AAAGTGAATA	AAGGCGGCCG	TTATCAAATG	CAGGTGGCAT	TGTGCAATGC	350
R-24	AAGGTGAATA	AAGGCGGCCG	TTATCAAATG	CAGGTGGCGT	TATGCAACGC	350
l-chi	CGACGGCTGT	ACCGCCAGCG	ATGCAACCGA	AATTGTGGTG	GCAGATACCG	400
m-chi	CGACGGCTGC	ACCGCCAGTG	ACGCCACCGA	AATTGTGGTG	GCCGACACCG	400
R-24	CGACGGCTGT	ACCGCCAGCG	ATGCAACCGA	AATTGTGGTG	GCAGATACCG	400
l-chi	ACGGTAGCCA	TTTGGCACCT	TTAAAAGAAC	CTTTGTTGGA	AAAGAATAAG	450
m-chi	ACGGCAGCCA	TTTGGCGCCG	TTGAAAGAGC	CGCTGCTGGA	AAAGAATAAA	450
R-24	ACGGTAGCCA	TTTGGCACCT	TTAAAAGAAC	CTTTGTTGGA	AAAGAATAAG	450
l-chi	CCTTATAAAC	AAGACTCCGG	CAAAGTGGTT	GGCTCTTATT	TCGTTGAATG	500
m-chi	CCGTATAAAC	AGAACTCCGG	CAAAGTGGTC	GGTTCTTATT	TCGTCGAGTG	500
R-24	CCTTATAAAC	AAGACTCCGG	CAAAGTGGTC	GGTTCTTATT	TCGTCGAGTG	500
l-chi	GGGCGTTTAC	GGCCGTAATT	TCACCGTCGA	TAAACTTCCG	GCTCAGAACC	550
m-chi	GGGCGTTTAC	GGGCGCAATT	TCACCGTCGA	CAAGATCCCG	GCGCAAAACC	550
R-24	GGGCGTTTAC	GGCCGTAATT	TCACCGTCGA	TAAACTTCCG	GCTCAGAACC	550
l-chi	TGACGCACCT	GCTGTACGGC	TTTATCCCTA	TCTGTGGCGG	TGACGGCATC	600
m-chi	TGACCCACCT	GCTGTACGGC	TTTATCCCGA	TCTGCGGCGG	CAATGGCATC	600
R-24	TGACGCACCT	GCTGTACGGC	TTTATCCCTA	TCTGTGGCGG	TGACGGCATC	600
l-chi	AACGACAGCC	TGAAAGAGAT	CGAAGGCAGC	TTCCAGGCGT	TACAGCGTTC	650
m-chi	AACGACAGCC	TGAAAGAGAT	TGAAGGCAGC	TTCCAGGCGT	TACAGCGCTC	650
R-24	AACGACAGCC	TGAAAGAGAT	TGAAGGCAGC	TTCCAGGCGT	TACAGCGCTC	650

FIG. 8(continued)

l-chi	CTGTCAGGGG	CGTGAAGACT	TTAAGGTATC	GATCCACGAT	CCGTTCGCTG	700
m-chi	CTGCCAGGGC	CGCGAGGACT	TCAAAGTCTC	GGTCCACGAT	CCGTTCGCCG	700
R-24	CTGCCAGGGC	CGCGAGGACT	TCAAAGTCTC	GGTCCACGAT	CCGTTCGCCG	700
l-chi	CGCTGCAGAA	AGGTCAGAAG	GGCGTGACCG	CCTGGGACGA	CCCCTACAAA	750
m-chi	CGCTGCAAAA	AGCGCAGAAG	GGCGTGACCG	CCTGGGATGA	CCCCTACAAG	750
R-24	CGCTGCAAAA	AGCGCAGAAG	GGCGTGACCG	CCTGGGATGA	CCCCTACAAG	750
l-chi	GGCAACTTCG	GCCAGTTGAT	GGCGTTGAAA	CAGGCGCGCC	CGGACCTGAA	800
m-chi	GGCAACTTCG	GCCAGCTGAT	GGCGCTGAAG	CAGGCGCATC	CTGACCTGAA	800
R-24	GGCAACTTCG	GCCAGCTGAT	GGCGCTGAAG	CAGGCGCATC	CTGACCTGAA	800
l-chi	AATCCTGCCG	TCGATCGGTG	GCTGGACGTT	ATCCGATCCG	TTCTTCTTTA	850
m-chi	AATCCTGCCG	TCGATCGGCG	GCTGGACGCT	GTCCGACCCG	TTCTTCTTCA	850
R-24	AATCCTGCCG	TCGATCGGCG	GCTGGACGCT	GTCCGACCCG	TTCTTCTTCA	850
l-chi	TGGGCGATAA	GGTGAAGCGC	GATCGCTTCG	TCGGCTCGGT	GAAGGAGTTC	900
m-chi	TGGGCGACAA	GGTGAAGCGC	GATCGCTTCG	TCGGTTCGGT	GAAAGAGTTC	900
R-24	TGGGCGACAA	GGTGAAGCGC	GATCGCTTCG	TCGGTTCGGT	GAAAGAGTTC	900
l-chi	CTGCAAACCT	GGAAGTTCTT	TGATGGCGTA	GATATCGACT	GGGAATTCCC	950
m-chi	CTGCAGACCT	GGAAGTTCTT	CGACGGCGTG	GATATCGACT	GGGAGTTCCC	950
R-24	CTGCAGACCT	GGAAGTTCTT	CGACGGCGTG	GATATCGACT	GGGAGTTCCC	950
l-chi	GGGCGGGCAG	GGTGCTAACC	CGAAACTGGG	CAGTACGCAG	GATGGGGCAA	1000
m-chi	GGGCGGCAAA	GGCGCCAACC	CTAACCTGGG	CAGCCCGCAA	GACGGGGAAA	1000
R-24	GGGCGGCAAA	GGCGCCAACC	CTAACCTGGG	CAGCCCGCAA	GACGGGGAAA	1000
l-chi	CCTATGTGCA	GCTGATGAAA	GAGCTGCGCG	CCATGCTGGA	TCAGCTTTCG	1050
m-chi	CCTATGTGTT	GCTGATGAAG	GAGCTGCGGG	CGATGCTGGA	TCAGCTGTCG	1050
R-24	CCTATGTGTT	GCTGATGAAG	GAGCTGCGGG	CGATGCTGGA	TCAGCTGTCG	1050
l-chi	GCGGAAACGG	GCCGTAAGTA	TGAACTGACC	TCTGCGATCA	GCGCCGGCAA	1100
m-chi	GCGGAAACCG	GCCGCAAGTA	TGAGCTGACC	TCCGCCATCA	GCGCCGGTAA	1100
R-24	GCGGAAACCG	GCCGCAAGTA	TGAGCTGACC	TCCGCCATCA	GCGCCGGTAA	1100
l-chi	GGATAAAATC	GATAAGGTGG	ATTACAACAC	CGCACAAAAC	TCGATGGATC	1150
m-chi	GGACAAGATC	GACAAGGTGG	CTTACAACGT	TGCGCAGAAC	TCGATGGATC	1150
R-24	GGACAAGATC	GACAAGGTGG	CTTACAACGT	TGCGCAGAAC	TCGATGGATC	1150
l-chi	ACATTTTCCT	GATGAGTTAC	GACTTCTATG	GGGCATTCGA	TCTGAAAAAT	1200
m-chi	ACATCTTCCT	GATGAGCTAC	GACTTCTATG	GCGCCTTCGA	TCTGAAGAAC	1200
R-24	ACATCTTCCT	GATGAGCTAC	GACTTCTATG	GCGCCTTCGA	TCTGAAGAAC	1200
l-chi	CTGGGCCACC	AGACTGCGCT	GAAAGCGCCG	GCCTGGAAAC	CGGATACGGC	1250
m-chi	CTGGGGCATC	AGACCGCGCT	GAATGCGCCG	GCCTGGAAGC	CGGACACCGC	1250
R-24	CTGGGGCATC	AGACCGCGCT	GAATGCGCCG	GCCTGGAAGC	CGGACACCGC	1250
l-chi	GTATACCACG	GTGAATGGCG	TTAATGCACT	GCTCACGCAG	GGCGTGAAGC	1300
m-chi	TTACACCACG	GTGAACGGCG	TCAATGCGCT	GCTGGCGCAG	GGCGTCAAGC	1300
R-241	TTACACCACG	GTGAACGGCG	TCAATGCGCT	GCTGGCGCAG	GGCGTGAAGC	1300

FIG. 8 (continued)

l-chi	CGGGCAAAAT	CGTGGTGGGC	ACCGCCATGT	ACGGTCGCGG	TTGGACCGGG	1350
m-chi	CGGGCAAGAT	CGTGGTCGGC	ACCGCCATGT	ATGGCCGCGG	CTGGACCGGG	1350
R-24	CGGGCAAAAT	CGTGGTGGGC	ACCGCCATGT	ACGGTCGCGG	TTGGACCGGG	1350
l-chi	GTGAACGGTT	ACCAGAACAA	CATTCCGTTT	ACCGGCACCG	CCACTGGCCC	1400
m-chi	GTGAACGGCT	ACCAGAACAA	CATTCCGTTc	ACCGGTACCG	CCACTGGGCC	1400
R-24	GTGAACGGTT	ACCAGAACAA	CATTCCGTTT	ACCGGCACCG	CCACTGGCCC	1400
l-chi	GGTGAAAGGC	ACCTGGGAAA	ATGGCATCGT	GGATTACCGC	CAGATCGCCA	1450
m-chi	GGTTAAAGGC	ACCTGGGAGA	ACGGCATCGT	GGACTACCGC	CAAATCGCCG	1450
R-24	GGTGAAAGGC	ACCTGGGAAA	ATGGCATCGT	GGATTACCGC	CAGATCGCCA	1450
l-chi	ATGAGTTTAT	GAGCGGCGAA	TGGCAGTACA	GCTACGATGC	TACCGCTGAA	1500
m-chi	GCCAGTTCAT	GAGCGGCGAG	TGGCAGTATA	CCTACGACGC	CACGGCGGAA	1500
R-24	ATGAGTTTAT	GAGCGGCGAA	TGGCAGTACA	GCTACGATGC	TACCGCTGAA	1500
l-chi	GCACCCTATG	TCTTCAAACC	TTCCACTGGC	GATCTGATCA	CCTTCGACGA	1550
m-chi	GCGCCTTACG	TGTTCAAACC	TTCCACCGGC	GATCTGATCA	CCTTCGACGA	1550
R-24	GCACCCTATG	TCTTCAAACC	TTCCACTGGC	GATCTGATCA	CCTTCGACGA	1550
l-chi	TGCGCGCTCG	GTGCAGGCGA	AGGGCAAATA	TGTGCTGGAT	AAGCAGCTGG	1600
m-chi	TGCCCGCTCG	GTGCAGGCCA	AAGGCAAGTA	CGTGCTGGAT	AAGCAGCTGG	1600
R-24	TGCGCGCTCG	GTGCAGGCGA	AGGGCAAATA	TGTGCTGGAT	AAGCAGCTGG	1600
l-chi	GCGGGTTGTT	CTCATGGGAA	ATTGACGCCG	ACAACGGCGA	TATTCTGAAT	1650
m-chi	GCGGCCTGTT	CTCCTGGGAG	ATCGACGCGG	ATAACGGCGA	TATTCTCAAC	1650
R-24	GCGGGTTGTT	CTCATGGGAA	ATTGACGCCG	ACAACGGCGA	TATTCTGAAT	1650
l-chi	AACATGAACA	GCAGCCTGGG	CAACAGCGTC	GGTACGCCTT	AA	1692
m-chi	AGCATGAACG	CCAGCCTGGG	CAACAGCGCC	GGCGTTCAAT	AA	1692
R-24	AACATGAACA	GCAGCCTGGG	CAACAGCGTC	GGTACGCCTT	AA	1692

FIG. 9

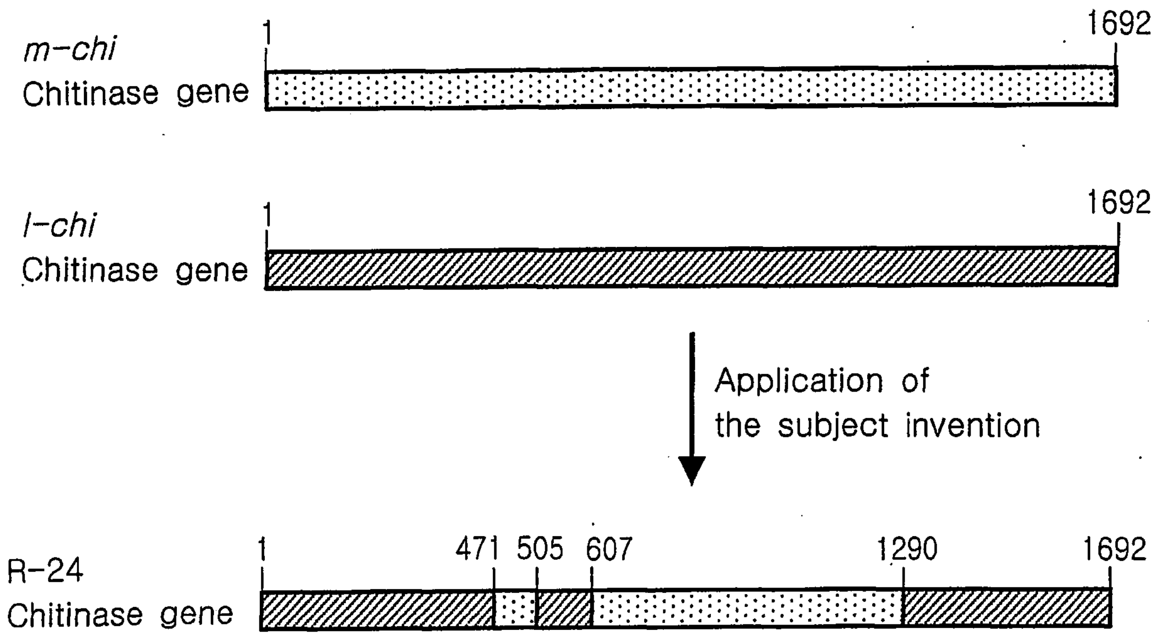
m-chi
Chitinase gene
1
1692
l-chi
Chitinase gene
1
1692
Application of
the subject invention
R-24
Chitinase gene
1
471
505
607
1290
1692

FIG. 10

Wild	ATGAATGGAAAAAGA	AAAATTTTCACATGT	ATTTCTATTGTAGGA	ATCGGACTAGCTAGT	60
M-13	ATGAATGGAAAAAGA	AAAATTTTCACATGT	ATTTCTATTGTAGGA	ATCGGACTAGCTAGC	60
M-20	ATGAATGGAAAAAGA	AAAATTTTCACGTGT	ATTTCTATTGTAGGA	ATCGGACTAGCTAGT	60
Wild	TTTTCTAATTCTAGT	TTCGCAGCAAGTGTA	ACGGACAATTCAGTA	CAAAATTCTATTCCC	120
M-13	TTTTCTAATTCTAGT	TTCGCAGCAAGTGTA	ACGGACAATTCAGTA	CAAAATTCTATTCCC	120
M-20	TTTTCTAATCCTAGT	TTCGCAGCAAGTGTA	ACGGACAATTCAGTA	CAAAATTCTATTCCC	120
Wild	GTAGTTAATCAACAA	GTAGCTGCTGCAAAG	GAAATGAAACCATTT	CCGCAGCAAGTTAAT	180
M-13	GTAGTTAATCAACAA	GTAGCTGCTGCAAAG	GAAATGAAACCATTT	CCGCAGCAAGTTTAT	180
M-20	GTAGTTAATCAACAA	GTAGCTGCTGCAAAG	GAAATGAAACCATTT	CCGCAGCAAGTTAAT	180
Wild	TATGCAGGTGTTATA	AAACCGAATCATGTT	ACACAGGAAAGTTTA	AATGCTTCTGTAAGA	240
M-13	TATGCAGGTGTTATA	AAACCGAATCATGTT	ACACAGGAAAGTTTA	AATGCTTCTGTAAGA	240
M-20	TATGCAGGTGTTATA	AAACCGAATCATGTT	ACACAGGAAAGTTTA	AATGCTTCTGTAAGA	240
Wild	AGTTACTACGATAAT	TGGAAAAAGAAATAT	TTGAAAAATGATTTA	TCTTCTTTACCTGGT	300
M-13	AGTTACTACGATAAT	TGGAAAAAGAAATAT	TTGAAAAATGATTTA	TCTTCTTTACCTGGT	300
M-20	AGTTACTACGATAAT	TGGAAAAAGAAATAT	TTGAAAAATGATTTA	TCTTCTTTACCTGGT	300
Wild	GGTTATTATGTAAAA	GGAGAGATTACAGGT	GATGCTGATGGGTTT	AAGCCACTTGGAACT	360
M-13	GGTTATTATGTAAAA	GGAGATATTACAGGT	GATGCTGATGGGTTT	AAGCCACTTGGAACT	360
M-20	GGTTATTATGTAAAA	GGAGATATTACAGGT	GATGCTGATGGGTTT	AAGCCACTTGGAACT	360
Wild	TCAGAAGGTCAAGGG	TATGGGATGATAATT	ACAGTATTAATGGCT	GGTTATGATTCGAAT	420
M-13	TCAGAAGGTCAAGGG	TATGGGATGATAATT	ACAGTATTAATGGCT	GGTTATGATTCGAAT	420
M-20	TCAGAAGGTCAAGGG	TATGGGATGATAATT	ACAGTATTAATGGCT	GGTTATGATTCGAAT	420
Wild	GCTCAAAAAATCTAT	GACGGTTTATTTAAA	ACAGCAAGAACTTTT	AAAAGTTCTCAAAAT	480
M-13	GCTCAAAAGATCTAT	GACGGTTTATTTAAA	ACAGCAAGAACTTTT	AAAAGTTCTCGAAAT	480
M-20	GCTCAAAAGATCTAT	GACGGTTTATTTAAA	ACAGCAAGAACTTTT	AAAAGTTCTCGAAAT	480
Wild	CCTAATTTAATGGGA	TGGGTTGTCGCAGAT	AGTAAAAAAGCACAA	GGTCATTTTGATTCT	540
M-13	CCTAATTTAATGGGA	TGGGTTGTCGCAGAT	AGTAAAAAAGCACAA	GGTCATTTTGATTCT	540
M-20	CCTAATTTAATGGGA	TGGGTTGTCGCAGAT	AGTAAAAAAGCACAA	GGTCATTTTGATTCT	540
Wild	GCTACTGATGGAGAT	TTAGATATTGCGTAT	TCTCTTCTTCTTGCT	CATAAGCAGTGGGGA	600
M-13	GCTACTGATGGAGAT	TTAGATATTGCGTAT	TCTCTTCTTCTTGCT	CATAAGCAGTGGGGA	600
M-20	GCTACTGATGGAGAT	TTAGATATTGCGTAT	TCTCTTCTTCTTGCT	CATAAGCAGTGGGGA	600
Wild	TCTAATGGAACAGTG	AATTATTTGAAAGAA	GCACAAGACATGATT	ACAAAAGGTATTAAA	660
M-13	TCTAATGGAACAGTG	AATTATTTGAAAGAA	GCACAAGACATGATT	ACAAAAGGTATTAAA	660
M-20	TCTAATGGAACAGTG	AATTATTTGAAAGAA	GCACAAGACATGATT	ACAAAAGGTATTAAA	660
Wild	GCTAGTAATGTTACA	AATAATAACCGACTA	AATTTAGGCGATTGG	GATTCTAAAAGTTCA	720
M-13	GCTAGTAATGTTACC	AATAATACCCGACTA	AATTTAGGCGATTGG	GATTCTAAAAGTTCA	720
M-20	GCTAGTAATGTTACA	AATAATAACCGACTA	AATTTAGGCGATTGG	GATTCTAAAAGTTCA	720

FIG. 10 (continued)

```
Wild  CTTGATACGAGACCA TCTGATTGGATGATG TCACACCTTAGAGCA TTTTATGAATTTACA  780
M-13  CTTGATACGAGACCA TCTGATTGGATGATG TCACACCTTAGAGCA TTTTATGAATTTACA  780
M-20  CTTGATACGAGACCA TCTGATTGGATGATG TCACACCTTAGAGCA TTTTATGAGTTTACA  780

Wild  GGTGATAAAACTTGG CTTACTGTTATTAAT AATTTGTACGATGTT TATACGCAATTTAGT  840
M-13  GGTGATAAAACTTGG CTTACTGTTATTAAT AATTTGTACGATGTT TATACGCAGTTTAGT  840
M-20  GGTGATAAAACTTGG CTTACTGTTATTAAT AATTTGTACGATGTT TATACGCAATTTAGT  840

Wild  AATAAGTACTCTCCA AATACAGGACTTATT TCAGATTTCGTTGTA AAAAACCCACCACAA  900
M-13  AATAAGTACTCTCCA AATACAGGACTTATT TCAGATTTCGTTGTA AAAAACCCACCACAA  900
M-20  AATAAGTACTCTCCA GATACAGGACTTATT TCAGATTTCGTTGTA AAAAACCCACCACAA  900

Wild  CCCGCACCTAAAGAC TTCTTAGAGGAGTCA GAATATACAAATGCA TATTATTACAATGCT  960
M-13  CCCGCACCTAAAGGC TTCTTAGGGGAGTCA GAATATACAAATGCA TATTATTACAATGCT  960
M-20  CCCGCACCTAAAGGC TTCTTAGGGGAGTCA GAATATACAAATGCA TATTATTACAATGCT  960

Wild  AGTCGGGTACCATTG AGAATTGTAATGGAC TATGCGATGTACGGC GAGAAAAGAAGTAAA  1020
M-13  AGTCGGGTACCATTG AGAATTGTAATGGAC TATGCGATGTACGGC GAGAAAAGAAGTAAA  1020
M-20  AGTCGGGTACCATTG AGAATTGTAATGGAC TATGCGATGTACGGC GAGAAAAGAAGTAAA  1020

Wild  GTCATTTCTGATAAA GTTTCTTCGTGGATT CAAAATAAAACGAAT GGAAATCCTTCTAAA  1080
M-13  GTCATTTCTGATAAG GTTTCTTCGTGGATT CAAAATAAAACGAAT GGAAATCCTTCTAAA  1080
M-20  GTCATTTCTGATAAG GTTTCTTCGTGGATT CAAAATAAAACGAAT GGAGATCCTTCTAAA  1080

Wild  ATTGTGGATGGTTAT CAATTAAATGGATCT AATATTGGTAGTTAT TCAACTGCTGTATTT  1140
M-13  ATTGTGGATGGTTAT CAATTAGATGGATCT AATATTGGTAGTTAT CCAACTGCTGTATTT  1140
M-20  ATTGTGGATGGTTAT CAATTAGATGGATCT GATATTGGTAGTTAT TCAACTGCTGTATTT  1140

Wild  GTTTCACCGTTTATT GCTGCAAGTATAACA AGTAGCAATAATCAA AAGTGGGTAAATAGC  1200
M-13  GTTTCACCGCTTATT GCTGCAAGTACAACA AGTAGCAATAATCAA AAGTGGGTAAATAGC  1200
M-20  GTTTCACCGTTTATT GCTGCAAGTATAACA AGTAGCAATAATCAA AAGTGGGTAAATAGC  1200

Wild  GGTTGGGATTGGATG AAGAATAAGAGAGAA AGTTATTTTAGTGAT AGTTATAATTTATTA  1260
M-13  GGTTGGGATTGGATG AAGAATAAGAGAGAA AGTTATTTTAGTGAT AGTTATAATTTATTA  1260
M-20  GGTTGGGATTGGATG AAGAATAAGAGAGAA AGTTATTTTAGCGAT AGTTATAATTTGTTA  1260

Wild  ACTATGTTATTCATT ACAGGAAATTGGTGG AAACCTGTACCTGAT GATACAAAAATACAA  1320
M-13  ACTATGTTATTCATT ACAGGGAATTGGTGG AAACCCGTACCTGGT GATACAAAAATACAA  1320
M-20  ACTATGTTATTCATT ACGGGAAATTGGTGG AAACCTGTACCTGAT GATACAAAAATACAA  1320

Wild  AATCAAATAAATGAT GCAATTTATGAAGGA TACGATAATTAA  1362
M-13  AATCAAATAAATGAT GCTATTTATGAAGGA TACGATAATTAA  1362
M-20  AATCAAATAAATGAT GCAATTTATGAAGGA TACGATAATTAA  1362
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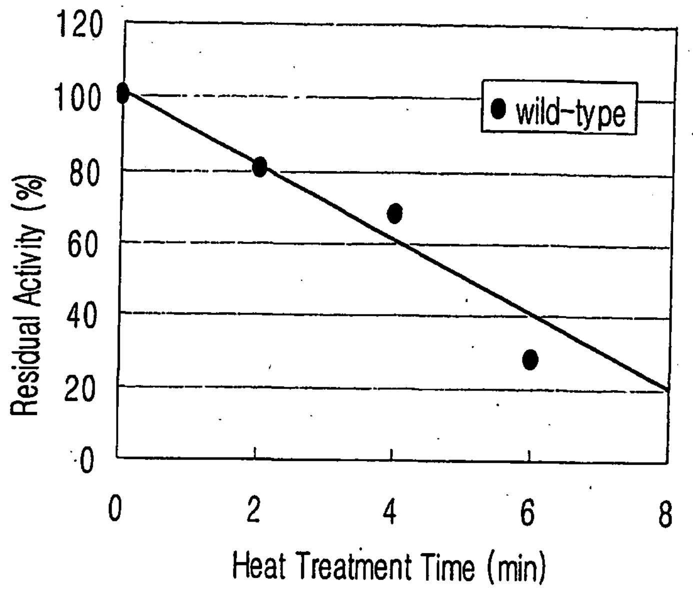
FIG. 11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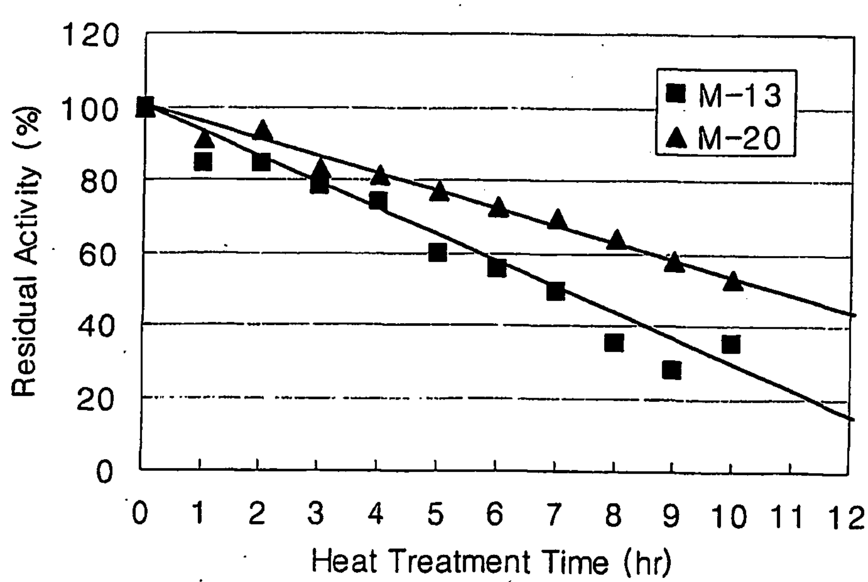
40
20
0
Residual Activity (%)
wild-type
0
2
4
6
8
Heat Treatment Time (min)


120
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80
60
40
20
0
Residual Activity (%)
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M-20
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1
2
3
4
5
6
7
8
9
10
11
12
Heat Treatment Time (hr)

SEQUENCE LISTING

<110> Amicogen, Inc.

<120> Method for generating recombinant DNA library using unidirectional single-stranded DNA fragments

<150> KR 2000-66889
<151> 2000-11-10

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<400> 2

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<212> DNA
<213> Artificial Sequence

<220>
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<212> DNA
<213> Artificial Sequence

<220>
<223> Chitinase recombinant DNA 2

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<212> DNA
<213> Artificial Sequence

<220>
<223> Chitinase recombinant DNA 3

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<211> 1692

<212> DNA

<213> Artificial Sequence

<220>

<223> Chitinase recombinant DNA 4

<400> 6

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ggtacgcctt aa 1692

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<211> 1692
<212> DNA
<213> Artificial Sequence

<220>
<223> Chitinase recombinant DNA 5

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ggcgttcaat aa 1692

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<211> 1692
<212> DNA
<213> Artificial Sequence

<220>
<223> Chitinase recombinant DNA 6

<400> 8

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ggtacgcctt aa 1692

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<212> DNA
<213> Artificial Sequence

<220>
<223> Chitinase recombinant DNA 7

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<212> DNA
<213> Artificial Sequence

<220>
<223> Chitinase recombinant DNA 8

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ggcgttcaat aa 1692

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<211> 1692
<212> DNA
<213> Artificial Sequence

<220>
<223> Chitinase recombinant DNA 9

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ggtacgcctt aa 1692

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<212> DNA
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<220>
<223> Chitinase recombinant DNA 10

<400> 12

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<211> 1692

<212> DNA

<213> Artificial Sequence

<220>

<223> R-24 chitinase gene

<400> 13

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ggtacgcctt aa 1692

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<211> 1362
<212> DNA
<213> Bacillus sp.

<400> 14

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<212> DNA
<213> Artificial Sequence

<220>
<223> M-13 mutant chitosanase gene

<400> 15

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<210> 16
<211> 1362
<212> DNA
<213> Artificial Sequence

<220>
<223> M-20 mutant chitosanase gene

<400> 16

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<220>
<223> Arbitrary polynucleotide

<400> 17

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<210> 18
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Arbitrary polynucleotide

<400> 18

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<210> 19
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> unidirectional DNA fragment

<400> 19

gaatgctaac tggacct 17

<210> 20
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<212> DNA
<213> Artificial Sequence

<220>
<223> Unidirectional DNA fragment

<400> 20

ctctctcaaa 10

<210> 21
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<400> 21

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<210> 22
<211> 20
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<223> Unidirectional DNA fragment

<400> 22

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<210> 23
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<212> DNA
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<220>
<223> Unidirectional DNA fragment

<400> 23
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<210> 24
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<400> 27

ccggaattcg tatgctaatt ccc 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01031

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
C12N 15/10, 15/00 : C12P 19/34 : C12Q 1/68 : G01N 33/566

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched
Korean Patents and Applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
Delphion, PAJ, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,994,368 (Syntex Inc.) 19 Feb 1991 See the abstract	1-17
A	US 5,811,238 (Affymax Technologies N.V.) 22 Sep 1998 See the abstract & Figures	1-17
A	US 5,962,272 (Clontech Lab. Inc.) 05 Oct 1999 See the abstract & Figures	1-17

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
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"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4,994,368	19 FEB 1991	EP 300796 B1	25 JAN 1989
		JP 2788034 B2	20 AUG 1998
		US 5273879 A	28 DEC 1993
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1995			
US 5,811,238	22 SEP 1998	EP 876509 A1	11 NOV 1998
		EP 911396 A2	28 APR 1999
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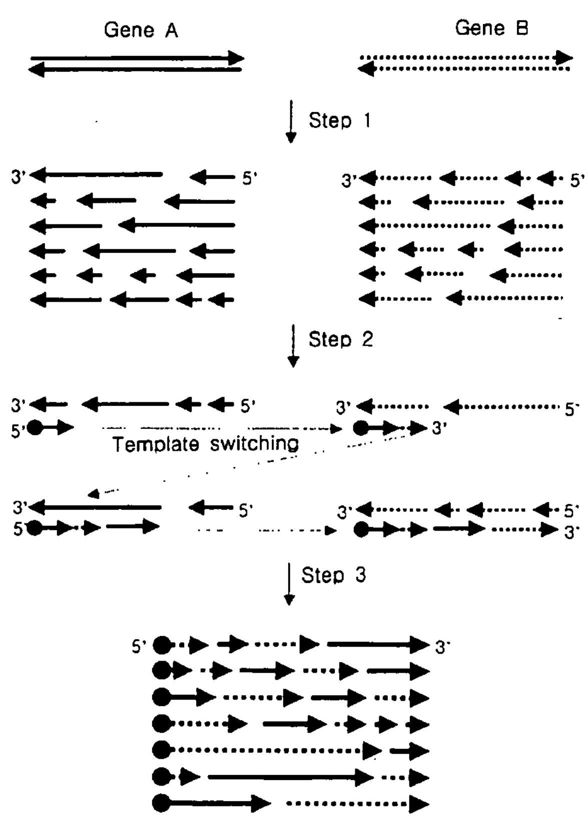
(54) **Title:** METHOD FOR GENERATING RECOMBINANT DNA LIBRARY USING UNIDIRECTIONAL SINGLE-STRANDED DNA FRAGMENTS



(57) **Abstract:** The present invention relates to a method for producing a recombinant polynucleotides comprising the steps of generating a pool of unidirectional single-stranded polynucleotide fragments from two or more homologous double-stranded polynucleotides, conducting a polymerization process comprising multi-cyclic extension reactions using the unidirectional single-stranded polynucleotide fragments as templates and specific oligonucleotides as primers to obtain recombinant polynucleotides, and conducting a polymerase chain reaction using at least one primer to amplify the recombinant polynucleotides; and a method for constructing a recombinant DNA library comprising the steps of inserting the recombinant polynucleotide prepared by the above method into a vector and transforming an expression cell with said vector containing the recombinant polynucleotide to obtain a plurality of mutant clones. The method of the present invention can be used for in vitro recombination of homologous polynucleotides and the directed molecular evolution.

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DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD FOR GENERATING RECOMBINANT DNA LIBRARY USING UNIDIRECTIONAL SINGLE-STRANDED DNA FRAGMENTS

FIELD OF THE INVENTION

The present invention relates to a method for the production of a pool of recombinant DNA encoding mutant proteins and a recombinant DNA library comprising same, which allows the directed evolution of proteins by *in vitro* recombination.

BACKGROUND OF THE INVENTION

Genetic information is eventually decoded into a protein which performs most of the vital functions in living organisms. As one of important biological macromolecules, protein not only serves as a component of cells but also participates in all the biochemical reactions with a high specificity.

The function of protein comprised of 20 kinds of amino acids is determined by the structure which is divided into four levels; primary, secondary, tertiary and quaternary structures. Since the primary structure of protein, i.e., amino acid sequence, especially contains the information regarding the shape and the function thereof, the whole structure or function of the protein can be changed even by a mutation in one amino acid residue (Shao, Z. and Arnold F.H., *Curr. Opin. Struct. Biol.* 6:513-518, 1996).

The diversity of organism reflects the diversity of genetic information encoded in DNA or RNA. In nature, the genetic information is changed slowly and continuously by a natural evolution process comprising mutation, sexual reproduction and natural selection. For example, during meiosis in sexual reproduction, homologous chromosomes derived from two individuals might exchange or reassemble their genetic materials through homologous recombination. Such reassembly of the DNA provides more chances for living organisms to expedite an evolution. However, it takes long time for

this type of evolution to occur in natural environment, partly due to its strong dependency on fortuity. Therefore, there have been many efforts to obtain, in a short period of time, a gene evolved for the desired purpose and a mutant protein by *in vitro* mutagenesis in combination with an appropriate screening method(Eigen, M., *Naturwissenschaften* 58:465-523, 1971; Bradt, R.M., *Nature* 317:804-806, 1985; Pal, K.F., *Bio. Cybern.* 69:539-546, 1993).

Current method in widespread use for creating mutant proteins is site-directed mutagenesis(Sambrook, J. *et al.*, *Molecular Cloning* 2nd, Cold Spring Harbor Lab Press, 1989). This method replaces nucleotides of desired site with a synthetically mutagenized oligonucleotide. However, there are limitations of the method in that it requires exact information on the amino acid sequence and the function of the site to be mutagenized in proteins. As another method for creating mutant proteins in a recombinant DNA library format, error-prone polymerase chain reaction(error-prone PCR) is used widely(Leung, D.W. *et al., Technique* 1:11-15, 1989; Caldwell, R.C. *et al., PCR Methods and Applications* 2:28-33, 1992). Error-prone PCR can be used for constructing a mutant DNA library of a gene by controlling the polymerization conditions to decrease the fidelity of polymerase. However, the error-prone PCR suffers from a low processibility of the polymerase, which limits the practical applications of the method for average-sized gene. Another limitation of error-prone PCR is that the frequency of co-occurrence of a plurality of mutations within a short-length region of DNA is too low for multiple mutations to be introduced.

To overcome said shortcomings of these methods, various methods for constructing a mutant DNA library from the mixture of homologous polynucleotides have been developed. Those are DNA shuffling method of Maxygen(USP 5,605,793; 6,117,679; 6,132,970), Gene Reassembly method of Diversa(USP 5,965,408) and recombination method developed by Frances H. Arnold(USP 6,153,410).

The DNA shuffling method of Maxygen, Inc.(USP 5,605,793; 6,117,679 and 6,132,970; Stemmer, W. P. C., *Nature*, *370*: 389-391, 1994; Stemmer, W. P. C., *Proc. Natl. Acad. Sci. USA*, *91*: 10747-10751, 1994)

comprises the steps of fragmenting at least one kind of double-stranded DNAs to be shuffled and conducting polymerase chain reactions(PCR) with the combined fragments, wherein the homologous fragments from different parent DNAs are annealed with each other to form partially overlapping DNA segments and DNA synthesis occurs by employing the respective DNA fragments as a template concurrently as a primer for each other to produce a random recombinant DNA library. However, this method requires a relatively large amount of DNA for preparing DNA fragments and DNase I used in the fragmentation process has to be removed from the resulting DNA fragments in an enough purity not to disturb subsequent polymerization process. Further, the application of the method is limited by the property of the DNase I. For example, DNase I widely used for the purpose is liable to cleave a 3'-phosphodiester bond having a pyrimidine base rather than a purine base at its terminus, which is a serious obstacle to get a completely randomized pool of DNA fragments(Shao, Z. *et al., Nucleic Acids Res.* 26:681-683, 1998).

Gene Reassembly method of Diversa Corporation(USP 5,965,408) comprises the steps of synthesizing DNA fragments by polymerization process employing at least one kind of double-stranded DNAs to be shuffled as templates and conducting polymerase chain reactions(PCR) with the combined fragments to produce a random recombinant DNA library. It employs partially synthesized fragments produced by UV treatment or adduct formation on the template DNA, thus preventing a complete polymerization on the template DNA. Despite of the randomness of the constructed DNA library, there are still problems for the method of Diversa Corporation in view of mutagenic potential of used reagents and tediousness to optimize the reaction conditions for the treatment of polymerization terminating reagent to obtain the desired size of fragments. In addition, when pyrimidine bases exist contiguously on the DNA strand, UV treatment induces pyrimidine dimers such as a thymidine dimer, which makes the template DNA distorted and prevent the progress of polymerase along with the strand. As a result, polymerizations are likely to end up at the site of pyrimidine dimer, thus DNA fragments obtained having insufficient randomness.

DNA shuffling and Gene Reassembly methods are characterized in that the formation of partially overlapping DNA segments is a prerequisite step and each DNA fragment derived from starting DNAs to be shuffled serves as not only a template but a primer.

Another method proposed by Arnold, staggered extension process(StEP)(USP 6,153,410; Zhao, H. *et al., Nat. Biotechnol.* 16:258-261, 1998; Encell, L.P. *et al., Nature Biotech.* 16:234-235, 1998) involves priming template double-stranded polynucleotides with random or specific primers, conducting PCR while controlling the reaction conditions to produce, in each cycle of reactions, short DNA fragments of staggered extension from the templates, and conducting repeated PCR to accomplish the recombination between genes by template switching. In case of polymerase reaction, there exist specific sequence-specific pause sites in each of target DNAs. In this line, StEP method has a problem in that the recombinant DNA library is biased from randomness since the extension rate of DNA fragments extended from the primers differs from each other even if the primers are annealed to the same region of different template DNAs(Encell, L.P. and Loeb, L. A., *Nature Biotech.*, *16*: 234-235 (1998)). In StEP method, PCR conditions have to be strictly controlled in order to get short DNA fragments from staggered extension of primers by shortening the polymerization time and lowering the reaction temperature. Failure to maintain the desirable range of temperature (e.g., too low temperature) during PCR process in StEP method may lead to non-specific annealing and further formation of undesirable recombinants.

A method for constructing a recombinant DNA library whereby said drawbacks of the conventional methods are overcome would be powerful for the production of mutant proteins having improved properties. The present invention described herein is directed to a method of *in vitro* recombination of heterologous DNA strands, which comprises preparing unidirectional single-stranded DNA fragments, mixing the DNA fragments with specific primers, followed by polymerization and further repeating the above steps to produce a recombinant DNA library. Further advantages of the present invention will

become apparent from the following description of the invention with reference to the attached drawings.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method for producing various recombinant polynucleotides through the random recombination between two or more homologous double-stranded polynucleotides.

Another object of the present invention is to provide a method for constructing a recombinant DNA library, which comprises the steps of inserting said recombinant polynucleotides into a vector and transforming an expression cell with the resulting vector to obtain a plurality of mutant clones.

A further object of the present invention is to provide a method for identifying an improved mutant gene by screening recombinant polynucleotides having a desired functional properties from said recombinant DNA library.

In accordance with one aspect of the present invention, there is provided a method for constructing a recombinant DNA library comprising the steps of:

(a) generating a pool of unidirectional single-stranded polynucleotide fragments randomized in length from two or more starting polynucleotides to be reassembled which have regions of similarity with each other;

(b) conducting a polymerization process comprising multi-cyclic extension reactions wherein the unidirectional single-stranded polynucleotide fragments prepared by step (a) serve only as templates and specific oligonucleotides are added to the reaction mixture as primers,

the primers being extended sequentially with directionality by means of template switching to produce at least one recombinant polynucleotide, and the resulting recombinant polynucleotide being

different from the starting polynucleotides in nucleotide sequence; and

(c) conducting a polymerase chain reaction using at least one specific primer to amplify the recombinant polynucleotides prepared by step (b).

In accordance with another aspect of the present invention, there is provided a method for constructing a recombinant DNA library, comprising the steps of inserting the recombinant polynucleotide prepared by the above method into a vector; and transforming an expression cell with said vector containing the recombinant polynucleotide to obtain a plurality of mutant clones.

In accordance with a further aspect of the present invention, there is provided a method for evolving a polynucleotide toward a desired property which comprises screening recombinant polynucleotides having a desired functional properties from the recombinant DNA library constructed by the above method.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows the schematic diagram illustrating the inventive method for constructing a recombinant DNA library employing unidirectional single-stranded polynucleotide fragments as templates for the polymerase chain reaction.

Fig. 2 compares the nucleotide sequences of the chitinase genes of *Serratia liquefaciens* (*l-chi*)(SEQ ID NO: 1) and *Serratia marcescens* (*m-chi*)(SEQ ID NO: 2). The corresponding bases of the two genes different from each other are marked by small letters.

Fig. 3 displays the result of 1% agarose gel electrophoresis, wherein

lane 1 of (a) is a standard DNA size marker(23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb from the top), lane 2 of (a), *in vitro* transcription product of chitinase gene of *Serratia marcescens*, lane 3 of (a), *in vitro* transcription product of chitinase gene of *Serratia liquefaciens*; lane 1 of (b) is a standard DNA size marker(23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb from the top), and lane 2 of (b), single-stranded DNA fragments produced by reverse transcription; and lane 1 of (c) is a standard DNA size marker(23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb from the top), and lane 2 of (c), PCR products produced by employing unidirectional single-stranded DNA fragments as templates.

Fig. 4 represents the result of 1% agarose gel electrophoresis of the digestion products obtained by extracting the plasmid DNAs from 14 clones randomly selected from the recombinant DNA library prepared by the inventive method and digesting the plasmid DNAs with restriction enzymes *Not*I, *Pst*I and *Hinc*II. Lane 1 is a standard DNA size marker and lanes 2 to 15, digestion products of the plasmid DNAs from the randomly selected 14 clones.

Fig. 5 compares the nucleotide sequences of the 10 recombinant DNAs (SEQ ID NOs: 3 to 12), which are randomly selected from the recombinant DNA library produced by the method of the present invention, with those of two wild-type genes, i.e., *l-chi* gene(SEQ ID NO: 1) and *m-chi* gene(SEQ ID NO: 2).

Fig. 6 is a schematic diagram showing the constitutions of the mutant recombinant DNAs of Fig. 5 in comparison with the two wild-type genes.

Fig. 7 shows the difference in the sizes of the clear zones made by the colonies expressing the recombinant chitinase genes on LB-agar plates containing 100 $\mu g/m\ell$ ampicillin and 0.5% swollen chitin, depending on the chitin decomposition capabilities of the colonies.

Fig. 8 compares the nucleotide sequence of R-24 chininase gene (SEQ ID NOs: 13) with those of two wild-type genes, i.e., *l-chi* gene(SEQ ID NO: 1) and *m-chi* gene(SEQ ID NO: 2).

Fig. 9 is a schematic diagram showing the constitution of R-24 chininase gene in comparison with the two wild-type genes.

Fig. 10 compares the nucleotide sequences of M-13 mutant(SEQ ID

NO: 15) and M-20 mutant(SEQ ID NO: 16) with that of wild-type chitosanase gene(SEQ ID NO: 14).

Fig. 11 depicts the differences in heat-stabilities of wild-type chitosanase derived from *Bacillus* sp. KCTC 0377BP, mutant M-13 and mutant M-20.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have endeavored to develop a new method for solving the problems of the prior art, and have accomplished the present invention by establishing a new method for producing a recombinant DNA library wherein a pool of various recombinant DNAs can be obtained more easily owing to the increased randomness introduced by a new principle different from those of the prior art.

The above-described DNA shuffling method of Maxygen, Inc.(US Patent Nos. 5,605,793; 6,117,679; and 6,132,970) and Gene Reassembly method of Diversa Corporation(US Patent No. 5,965,408) are commonly characterized in that the double-stranded DNA fragments obtained from more than two polynucleotides to be reassembled are converted to single stands and then annealed with each other to form partially overlapping DNA segments, and, accordingly, they are used as primers as well as templates for nucleotide extension in the polymerase chain reaction(US Patent Nos. 4,683,202 and 4,683,195) and elongated by repeating identical multi-cyclic polymerization reactions. In contrast, the method of the present invention is basically different from the prior art in that the unidirectional single-stranded polynucleotide fragments derived from two or more polynucleotides to be reassembled are used and, accordingly, no partially overlapping DNA segments are formed within the pool of single-stranded polynucleotide fragments and the unidirectional polynucleotide fragments serve only as templates; that just the oligonucleotides added as primers are elongated gradually with a directionality using the unidirectional single-stranded polynucleotide fragments as templates; and that recombination is introduced

by template switching during this PCR process. Further, unlike the Arnold's StEP method(US Patent No. 6,153,410) which employs the stringent conditions controlling temperature and reaction time to produce partially elongated DNA fragment from the double-stranded target DNA used as a template, the method of the present invention uses DNA fragments as templates and, therefore, DNA fragments elongated as long as the template DNA fragments can be obtained by employing a conventional condition of polymerization reaction. Further, it is possible to increase the randomness of recombination significantly since the inventive method is not influenced by the delayed elongation rate of polymerase at the sequence-specific pause sites.

The method of the present invention for producing mutant recombinant polynucleotides provides a method for producing a group of various recombinant genes by exchanging parts of two or more homologous genes with each other, and comprises the steps of:

(a) generating a pool of unidirectional single-stranded polynucleotide fragments randomized in length from two or more starting polynucleotides to be reassembled which have regions of similarity with each other;

(b) conducting a polymerization process comprising multi-cyclic extension reactions wherein the unidirectional single-stranded polynucleotide fragments prepared by step (a) serve only as templates and specific oligonucleotides are added to the reaction mixture as primers, the primers being extended sequentially with directionality by means of template switching to produce at least one recombinant polynucleotide, and the resulting recombinant polynucleotide being different from the starting polynucleotides in nucleotide sequence; and

(c) conducting a polymerase chain reaction using at least one specific primer to amplify the recombinant polynucleotides prepared by step (b).

In the polymerization reaction of step (b), when the partially elongated DNA fragments from specific primers are annealed with the template DNA

fragments originated from the other starting double-stranded polynucleotide in the next cycle and the polymerization reaction is progressed, then recombinant polynucleotides containing the sequences originating from the two homologous polynucleotides in a polynucleotide are resulted therefrom. By repeating such PCR cycles, it is possible to obtain various mutant recombinant polynucleotides having randomly reassembled sequences between A and B gene as shown in Fig. 1.

In addition, the present invention provides a method for constructing a recombinant DNA library, comprising the steps of inserting the recombinant polynucleotide prepared as above into a vector; and transforming an expression cell with said vector containing the recombinant polynucleotide to obtain a plurality of mutant clones.

It is possible to screen a useful gene from the recombinant DNA library constructed by the inventive method.

Accordingly, the present invention further provides a method for identifying an improved mutant gene, which comprises screening recombinant polynucleotides having a desired functional property from the recombinant DNA library constructed by the above method.

The present invention relates to a method for producing a recombinant DNA library by random recombination between two or more genetic materials. According to the present invention, it is possible to synthesize various kinds of recombinant genes by *in vitro* random recombination and to prepare a novel polypeptide having a desired property by screening a clone having a desired gene from a recombinant DNA library constructed by using the recombinant genes together with a suitable expression vector and a host cell and expressing the polypeptide therefrom.

As used herein, the term "unidirectional single-stranded DNA or polynucleotide fragments" means that the single-stranded DNA or polynucleotide fragments are not anti-parallel, but parallel to each other and, accordingly, they cannot anneal with each other via complementary hydrogen bonds even if they are mixed together. For instance, when the entire nucleotide sequence of a double-stranded DNA is as follows,

5'-AGGTCCAGTTAGCATTCGGAAAGGCCGTTTGAGAGAG-3' (SEQ ID NO: 17)

3'-TCCAGGTCAATCGTAAGCCTTTCCGGCAAACTCTCTC-5' (SEQ ID NO: 18)

the single-stranded DNAs derived therefrom such as 3'-TCCAGGTCAATCGTAAG-5'(SEQ ID NO: 19), 3'-AAACTCTCTC-5'(SEQ ID NO: 20), 3'-TTTCCGGCAAACTCTCTC-5'(SEQ ID NO: 21), 3'-CCTTTCCGGCAAACTCTCTC-5'(SEQ ID NO: 22) and 3'-TCAATCG TAAGCCTTTCCGGCAAACTCTCTC-5'(SEQ ID NO: 23) are considered to be unidirectional. Such unidirectional single-stranded DNA or polynucleotide fragments, which are employed in the method of the present invention only as templates for polymerase chain reactions, may be prepared to have various lengths depending on the sizes of the polynucleotides to be reassembled.

The term "recombinant DNA" as used herein means a chimeric DNA of a nucleotide sequence mosaic including nucleotide sequences originating from two or more polynucleotides, which are substantially homologous but not identical, in a molecule. The chimeric DNA contains a region of original nucleotide sequence and another region of mutated nucleotide sequence. Figs. 5 and 6 illustrates such recombinant DNAs synthesized by the random *in vitro* DNA recombination by the method of the present invention. Unlike the recombinant DNA naturally produced by the gene exchange due to the crossing over between homologous chromosomes in the meiosis during sexual reproduction, the recombinant DNAs of the inventive method is produced to have various nucleotide sequences in a short time by the *in vitro* random recombination between homologous DNA strands and they can be inserted into a vector and expressed in a host cell transformed by the vector. A recombinant DNA library consisting of clones containing various recombinant DNAs can be constructed and a recombinant DNA having a desired property can be screened therefrom. As discussed above, the combination of *in vitro* production of random recombinant DNA library between two or more homologous polynucleotides with a screening technique mimicking the natural selection has an advantage in that an improved gene or mutant protein having a desired property can be obtained in a short time.

As used herein, the term "homologous" means that one single-stranded

nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the nucleic acid sequences and the hybridization conditions such as temperature and salt concentration.

As used herein, the term "mutation" means changes in the sequence of a wild-type nucleic acid sequence or changes in the sequence of a peptide expressed therefrom.

As used herein, the term "DNA library" means a set of polynucleotides or recombinant DNA fragments each consisting of two or more polynucleotides and produced by random recombination. The DNA library includes: a set of polynucleotides having various nucleotide sequence; a sum of DNAs having various nucleotide sequences or cloned DNAs; or, in a broad sense, a set of clones containing said DNAs. A recombinant DNA encoding a protein having a desired property can be screened from such DNA library and used for protein expression.

More specifically, the present invention provides a method for producing recombinant polynucleotides having randomly and artificially mutated various nucleotide sequences from naturally existing or artificially prepared two or more homologous polynucleotides by the following steps. Fig. 1 illustrates this *in vitro* DNA recombination method.

Step 1: A set of unidirectional single-stranded polynucleotide fragments of random lengths are generated from two or more starting polynucleotides to be reassembled, wherein the starting polynucleotides have regions of similarity with each other(Step 1 of Fig. 1).

The starting polynucleotides for use in the present invention may have a homology of more than 50% with each other, and it is preferred to employ starting polynucleotides having homologies of more than 80%.

All of the single-stranded polynucleotide fragments produced from two or more homologous polynucleotides have identical unidirectional properties. Therefore, they are parallel to each other and, accordingly, a complementary

annealing between them through complementary hydrogen bonds cannot occur even if they are mixed together.

The unidirectional single-stranded polynucleotide fragments can be prepared by any one of conventional methods, e.g., a method producing unidirectional single-stranded polynucleotide fragments from RNA by reverse-transcription, a method for producing single-stranded polynucleotide fragments by gradual unidirectional deletion of nucleotides, a method for producing single-stranded polynucleotide fragments from complementary single-stranded polynucleotides. The single-stranded polynucleotide fragments can be prepared from RNA or single-stranded DNA beginning with random primers(Feinberg, A. P. and Vogelstein, B., *Anal. Biochem.*, *132*: 6-13 (1983)) by employing reverse transcriptase(Gerard, G. F. et al., *Mol. Biotechnol.*, *8*: 61-77 (1997)), bacteriophage T4 DNA polymerase(Nossal, N. G., *J. Biol. Chem.*, *249*: 5668-5676 (1974)), bacteriophage T7 DNA polymerase(Tabor, S. and Richardson, C. C., *J. Biol. Chem.*, *264*: 6447-6458 (1989)), Klenow enzyme(Klenow, H. and Henningsen, I., *Proc. Natl. Acad. Sci. USA*, *65*: 168 (1970)), etc. At this time, the size of single-stranded polynucleotide can be regulated by controlling the concentration of random primers or adding an appropriate concentration of dideoxynucleotides(2',3'-dideoxyadenosine 5'-triphosphate, 2',3'-dideoxyguanosine 5'-triphosphate, 2',3'-dideoxycytidine 5'-triphosphate, 2',3'-dideoxythymidine 5'-triphosphate) to the reaction mixture to obtain single-stranded polynucleotide fragments of which length is gradually elongated from the random primers. The single-stranded polynucleotide fragments having gradual unidirectional deletions of nucleotides may be obtained by employing exonucleases capable of successively digesting the nucleotides from the 5' end of a single-stranded polynucleotide.

More specifically, the unidirectional single-stranded polynucleotide fragments can be prepared by any one of the following processes:

A process comprising the steps of (i) conducting a transcription process to produce RNA from at least one starting polynucleotide; and (ii) conducting a reverse transcription process, wherein random primers are used as primers

and the RNA transcript of step (i) as a template;

A process comprising the steps of (i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme; (ii) producing a pool of double-stranded polynucleotides having unidirectional sequential deletion by treating the reaction mixture of step (i) with exonuclease III followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease III; (iii) treating the resulting double-stranded polynucleotides having a 5'-overhang with an S1 nuclease and a DNA polymerase to form a blunt end thereof; (iv) generating a new 3'-overhang to the same side which has 3'-overhang in step (i); and (v) treating the polynucleotides of step (iv) with exonuclease III to generate single-stranded polynucleotides;

A process comprising the steps of (i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme; (ii) treating the polynucleotides of step (i) with exonuclease III to generate single-stranded polynucleotides; and (iii) conducting a polymerization process on the single-stranded polynucleotides of step (ii) using random primers;

A process comprising the steps of (i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme; (ii) treating the polynucleotides of step (i) with exonuclease III to generate single-stranded polynucleotides; and (iii) producing a pool of single-stranded polynucleotides having unidirectional sequential deletion by treating the single-stranded polynucleotides of step (ii) with a single-strand specific 5'→3' exonuclease followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease.

A process comprising the steps of (i) conducting a polymerase chain reaction on the starting double-stranded polynucleotides using only one kind of oligonucleotide among forward and reverse primers; (ii) isolating the

resulting single-stranded polynucleotides from the starting double-stranded polynucleotides; and (iii) conducting a polymerization process on the single-stranded polynucleotides of step (ii) using random primers;

A process comprising the steps of (i) conducting a polymerase chain reaction on the starting double-stranded polynucleotides using only one kind of oligonucleotide among forward and reverse primers; (ii) isolating the resulting single-stranded polynucleotides from the starting double-stranded polynucleotides; and (iii) treating the single-stranded polynucleotides of step (ii) with a single-strand specific 5'→3' exonuclease followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease; and

A process for preparing the steps of (i) isolating a single-stranded polynucleotide from a viral vector or plasmid vector which has at least one starting polynucleotide insert; and (ii) conducting a polymerization process on the single-stranded polynucleotides of step (i) using random primers.

Step 2: The second step of the inventive method may comprise the steps of (i) conducting at least one cycle wherein the primers are extended to the end of the unidirectional single-stranded DNA fragments used as templates; (ii) conducting at least one subsequent cycle wherein each of the resulting extended polynucleotides of step (i) is further extended to the end of an unidirectional single-stranded DNA fragment other than the unidirectional single-stranded DNA fragment used in step (i) by means of template switching; and (iii) repeating step (ii) until recombinant polynucleotides of desired length are obtained.

Specifically, the unidirectional single-stranded polynucleotide fragments of various lengths prepared in Step 1 are mixed together, a specific oligonucleotide having a nucleotide sequence complementary to the single-stranded polynucleotide fragments are added thereto, and a polymerase chain reaction is carried out under a proper stringency. Then, the specific oligonucleotide serves as a primer of polymerase chain reaction and is

elongated gradually at one direction(5'→3') in each turn of reactions, whereby the recombination reaction occurs. The synthesized polynucleotides are separated into single strands by denaturation process and re-annealed. At this time, the synthesized polynucleotide may be annealed with other polynucleotide fragment containing a homologous sequence.

More specifically, a mixture of double-stranded polynucleotides can be denatured by heat and consequent polymerase chain reaction consists of the following three steps. First, double-stranded template DNA is treated at 90 to 98℃ for 10 sec to 5 min in order to separate into single-strands(denaturation). Thereafter, by lowering the temperature, previously added primers are annealed with a complementary single-stranded template DNA(annealing). This step is carried out at 40 to 72℃ for 10 sec to 2 min. Then, upon regulation of the temperature within a range of 70 to 78℃, four kinds of dNTPs(dATP, dGTP, dCTP, dTTP) in the reaction mixture begin to react and a DNA complementary to the template DNA is synthesized and elongated. The reaction time depends on the length of DNA being synthesized.

In case of producing various recombinant DNAs in such a manner from two or more polynucleotides having homologous nucleotide sequences, a polynucleotide may extend from an oligonucleotide primer, which is capable of hybridizing with at least one of the starting polynucleotides, up to the 5' end of the unidirectional single-stranded DNA fragment used as a template in a cycle of synthesis; and the resulting polynucleotide may further extend to the end of other unidirectional single-stranded polynucleotide originating from other starting polynucleotide by template switching in the next cycle. At this time, a recombination boundary is formed between the oligonucleotides synthesized by employing as templates unidirectional single-stranded polynucleotides originating from different starting polynucleotides.

In Step 2 for the extension of polynucleotide, the unidirectional single-stranded polynucleotide fragments prepared in Step 1 are employed only as templates for generating the recombinant DNAs and, accordingly, the primers added at the begining are extended gradually to one direction(5'→3') using

them as templates through the repetitive PCR to result in generation of recombinant polynucleotides.

In Step 2, the DNA recombination is conducted by periodically repeating the steps of denaturation, annealing and extension at the presence of DNA polymerase for the desired period. The degree of recombination depends on the homology between the groups of single-stranded polynucleotides derived from different starting polynucleotides.

Step 3: By sufficiently repeating the PCR cycles of Step 2 and amplifying the resulting mutant recombinant polynucleotides by a normal PCR method, a recombinant double-stranded DNA library is prepared. The recombinant DNA library thus obtained may consist of various kinds of mutant double-stranded polynucleotides which contain in a molecule the identical and heterogenous regions as compared with corresponding regions of any one of the starting double-stranded polynucleotides. The nucleotide sequence of the recombinant DNA may be determined by a conventional method, e.g., Maxam-Gilbert's method(Maxam, A. M and Gilbert, W., *Mol. Biol.(Mosk)*, *20*: 581-638 (1986)), Dideoxy method(Messing, J. et al., *Nucleic Acids Res.*, *24*; 309-321(1981)), or a method using DNA fluorescence marker and automated DNA sequence analyzer.

The present invention further provides a method for constructing a recombinant DNA library for screening a desired gene using the recombinant DNAs obtained by the above method. Specially, it comprises the steps of inserting the mutant recombinant double-stranded DNA obtained in Step 3 into an appropriate expression vector, introducing the resulting expression vector into an expression cell to obtain a library containing a plurality of clones; screening a desired polynucleotide from the clones; and expressing a protein from the polynucleotide by a conventional method. Suitable expression methods include: producing and accumulating a gene product in cells; secreting a gene product from a cell and accumulating them in a medium; secreting a gene product into a periplasm; and the like methods. For

screening a desired gene product from a recombinant DNA library, the methods known in the art, e.g. immunochemical method, radiochemical method, a method employing surface expressing system, and gene chip screening method, may be employed alone or in combination. In preparing the recombinant DNA library, any expression vector that operates in a selected host cell may be employed, exemplary vectors including conventional vectors of phage, plasmid, phagemid, viral vector and artificial chromosome known in the art. The method for constructing the expression vector is well known in the art, e.g., in Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual,* 2nd ed., (1989) Cold Spring Harbor Laboratory Press, N.Y. A suitable host cell may be transformed with the resulting expression vector. The suitable host cells for expressing the recombinant DNA include a bacterium such as *E. coli*, *Bacillus subtilis* and *B. brevis*, etc.; an Actinomyces such as *Streptomyces lividans*; a yeast such as *Saccharomyces serevisiae*; a fungus such as *Aspergillus oryzae*, *A. nidulans* and *A. niger*; an animal cell such as COS-7, CHO, Vero and mouse L cells; an insect cell; and a plant cell.

The present invention provides a method for preparing various, random, mutant recombinant DNAs in a short period of time. Specifically, a library of mutant recombinant polynucleotides can be obtained by adding oligonucleotide primers to a mixture of unidirectional single-stranded DNA fragments derived from two or more of homologous nucleic acid sequences or polynucleotides; and conducting repetitive PCR to obtain the library of mutant recombinant polynucleotides, wherein random recombinations between the nucleotide sequences of the single-stranded oligonucleotide fragments are occurred.

The recombinant DNAs prepared by the inventive method may be genes encoding proteins, e.g., enzymes, antibodies, vaccines(antigens), hormones, growth factors, binding proteins and plasma proteins. For instance, the recombinant DNA may encode an enzyme, said enzyme being selected from the group consisting of hydrolase, lyase, transferase, oxidoreductase, ligase and isomerase. A preferred embodiment of the present invention

provides a method for constructing a recombinant DNA library by preparing a recombinant gene(recombinant DNA) having a random mutation between *Serratia marcescens* chitinase gene(SEQ ID NO: 1, designated "*m-chi*") and *S. liquefaciens* chitinase gene(SEQ ID NO: 2, designated "*l-chi*") and cloning the recombinant gene. About 10,000 clones were prepared by the inventive method and, among them, 10 clones were randomly selected to determine the nucleotide sequences thereof. Comparison of their nucleotide sequences with those of the two wild-type genes exhibited that one time of recombination is occurred between the two genes in recombinant clones 3, 4 and 10; two times of recombinations, in recombinant clones 1, 2, 7 and 8; three times of recombinations, in recombinant clones 6 and 9; and four times of recombinations, in reocmbinant clone 5. These results demonstrate that the inventive method is effective in constructing a recombinant DNA library having a random recombination between two or more kinds of polynucleotides.

The inventive method for constructing a recombinant DNA library has a wide applicability. This *in vitro* mutagenization method may be used in a laboratory as means for biochemical studies. Since it allows to understand the mechanism of a protein involving in the maintenance and regulation of life in a molecular level, it may be used as means for producing and screening a proten such as an enzyme, antibody, vaccine(antigen), hormone, adsorption protein or plasma protein, thereby inducing the change of substrate specificity, change of reaction specificity, increase of activity, change of antigenicity, change of safety of a protein. Therefore, it is ultimately applied to various industrial fields for the development of a medicine, improvement and enhancement of food quality, improvement of energy conversion rate, breeding and quality improvement in livestock and fishery, development and production of novel chemical product, etc.(Chartrain M. et al., *Curr. Opin. In Biotech.*, *11*: 209-214 (2000); Miyazaki K. et al., *J. Mol. Biol.*, *297*: 1015-1026 (2000); Giver, L. and Arnold, F. H., *Curr. Opin. Chem. Biol.*, *2*: 335-338 (1998); Kumamaru, T. et al., *Nat. Biotechnol.*, *16*: 663-666 (1998); and Patten, P. A., *Curr. Opin. Biotechnol.*, *8*: 724-733 (1997)).

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usage and conditions.

Example 1 : Generating unidirectional single-stranded polynucleotide fragments

A pool of unidirectional single-stranded polynucleotide fragments having random length was prepared from a pair of double-stranded polynucleotides which have regions of similarity with each other as follows:

1-1) Preparation of unidirectional single-stranded DNA fragments by Reverse Transcription

In an embodiment of the present invention, genes encoding chitinase of *Serratia marcescens* and *Serratia liquefaciens* (hereinafter, referred to as "*m-chi*" and "*l-chi*", respectively) were chosen as starting polynucleotides to be reassembled, the nucleotide sequences of which are described in Fig. 2.

*Hin*dIII/*Xba* I fragments containing *m-chi* and *l-chi* genes, respectively, were cloned into pUC19, resulting in pUC19-*m-chi* and pUC19-*l-chi*. These plasmids were treated with *Nde* I , gap-filled with Klenow and then digested with *Hin*dIII. The resulting DNA inserts of about 2-kb were ligated to the *Hin*dIII/*Eco*RV backbone of pBluescript II KS (Stratagene) to give 5-kb recombinant plasmids. The resulting plasmids, pBSK-*m-chi* and pBSK-*l-chi*, were then linearized with *Spe* I .

200 ng of the linearized plasmids was added to transcription buffer solution [40 mM Tris-HCl(pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT] supplemented with 0.5 mM each rNTP, 40 units of RNasin and 17 units of T3 RNA polymerase up to the total volume of 20$\mu\ell$

and incubated at 37℃ for 1 hour. The RNA transcripts of the *m-chi* and *l-chi* genes obtained by the above *in vitro* transcription were analyzed by electrophoresis on 1% agarose gel. The bands of 5-kb plasmid and RNA transcripts are detected in lanes 2 and 3 of Fig. 3 (a). Purified with RNAeasy column(Qiagen), 200 ng of each RNA transcribed from the two chitinase genes was mixed. The RNA mixture was added to the reaction buffer[10 mM Tris-HCl(pH 8.3), 15 mM KCl, 0.6 mM $MgCl_2$, 0.2 mM DTT] supplemented with 6 μg of random hexamer (Genotech, Inc.), 0.2 mM each dNTP, 40 units of RNasin and 50 units of M-MLV reverse transcriptase to the total volume of 50 μl and reverse transcription was performed at 37℃ for 1 hour. After the reverse transcription, the RNA templates were removed by incubating the reaction mixture with 20 ng of RNase I at 37℃ for 1 hour.

Since the random hexamer can be hybridized with the template RNA at all the location thereof by chance, nucleotide extension from the random hexamer generates unidirectional single-stranded DNA fragments with random length.

The products of reverse transcription were electrophorezed on 1% agarose gel (lane 2, Fig. 3 (b)) and the single-stranded DNA fragments were cut and purified using a Geneclean kit (Bio 101).

<u>1-2) Preparation of unidirectional single-strand DNA fragments with serial 5' deletions</u>

This method is based on two useful features of exonuclease III: (i) processive digestion at a very uniform rate and (ii) failure to initiate digestion at DNA ends with 4-base 3'-protrusions (Henikoff, S., *Gene* 28, 351-359, 1984).

Plasmid pGEM-T (Promega) having *m-chi* gene of 30μg was linearized with a pair of restriction enzymes, *Sph* I and *Nco* I, wherein *Sph* I produces 4-base 3'-protrusions resistant to the exonuclease III digestion while *Nco* I generates 4-base 5'-overhanging ends. As for *l-chi* gene, the above process was conducted as same. The linearized polynucleotides dissolved in exonuclease III reaction buffer[66 mM Tris-HCl (pH 8.0), 0.66

mM $MgCl_2$] up to the volume of 60μl were digested with 2 units of exonuclease Ⅲ. 2.5μl of aliquot was then removed at intervals of twenty seconds, and the enzyme reaction was terminated. The resulting aliquot was mixed with 7.5μl of S1 nuclease mix [S1 nuclease reaction buffer(300 mM potassium acetate, pH 4.6, 2.5 M NaCl, 10 mM $ZnSO_4$, 50% glycerol) plus 50 units of S1 nuclease] and then placed at room temperature for 15 minutes.

After the S1 nuclease was inactivated by S1 stop solution[300 mM Tris base, 50 mM EDTA], polymerization was performed at 37℃ for 30 min by adding Klenow and then the products were cleaved with *Sac* I. The resulting double-stranded DNA fragments having random deletions sequentially were analyzed by electrophoresis on 1% agarose gel. The DNA fragments were extracted from the gel and reacted with 2 units of exonuclease Ⅲ for 1 hour to produce a set of single-stranded DNA fragments having unidirectional deletions thereon.

<u>1-3) Preparation of unidirectional single-strand DNA fragments using single-stranded DNA as a template</u>

Plasmid pGEM-T (Promega) having each of *m-chi and l-chi* genes of 5 μg was linearized with a pair of restriction enzymes, *Sph* I and *Nco* I. The linearized polynucleotides dissolved in exonuclease Ⅲ reaction buffer[66 mM Tris-HCl (pH 8.0), 0.66 mM $MgCl_2$] up to the volume of 60μl were digested with 2 units of exonuclease Ⅲ at 37℃ for 30 min.

The resulting linearized single-stranded polynucleotides were used as templates to generate the single-stranded DNA fragments in a polymerization mix[10 units of Klenow, 6μg of random hexamers, 0.1 mM each dNTP, 10 mM Tris- HCl(pH 7.5), 5 mM $MgCl_2$, 7.5 mM DTT] at 37℃.

The resulting unidirectional single-stranded DNA fragments were analyzed by electrophoresis on 1% agarose gel and subsequently purified using a Geneclean kit(Bio 101).

Example 2 : Reassembly of polynucleotides by polymerase chain reaction using the unidirectional single-stranded DNA fragment as a template

The unidirectional single-stranded DNA fragments obtained above served as templates for polymerase chain reaction. A reaction mixture contained 20 ng of the single-stranded DNA fragments, 0.2 mM each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl(pH 8.8), 0.1% Triton X-100, 2 units of Vent DNA polymerase (New England BioLabs) and 25 pmole of a primer in a total volume of 50$\mu\ell$, wherein the primer being an oligonucleotide(SEQ ID NO: 24) having the nucleotide sequence identical to those at the 5' termini of *m-chi* and *l-chi* genes. PCR was carried out on an MJ Research thermal cycler (PTC-100) at 94℃ for 3 min; 94℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds (30 cycles); and 72℃, 5 min. For the amplification of a full-length DNA, secondary PCR was carried out on the above PCR products using 25 pmole of a 3'-specific oligonucleotide (SEQ ID NO: 25) as a primer. PCR was carried out on an MJ Research thermal cycler (PTC-100) at 94℃ for 3 min; 94℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds (30 cycles); and 72℃, 5 min. The resulting PCR products of about 1.7 kb were analyzed by 1% agarose gel electrophoresis (lane 2, Fig. 3 (c)).

Example 3 : Sequencing and screening

The PCR products of example 2 were extracted from the gel by a Geneclean kit(Bio 101), digested with *Hin*dIII and *Xba* I , and ligated to the *Hin*dIII/*Xba* I backbone of pBluescriptII KS. The resulting recombinant plasmid was transformed to *E. coli* JM83 and transformants were selected on LB-agar plates supplemented with 100μg/ml ampicillin. Plasmid DNA was isolated from the randomly chosen 14 colonies by Qiagen Spin Miniprep kit(Qiagen) and digested with restriction enzymes, *Not* I , *Pst* I and *Hinc* II .

Fig. 4 shows various sizes of DNA resolved by usual electrophoresis on 1% agarose gel. The band patterns of DNA fragments of *l-chi* gene cleaved with the same three restriction enzymes are shown in lane 5, those of *m-chi* gene in lanes 8 and 13. The remaining lanes represent patterns of random recombinant DNA reassembled from *m-chi* and *l-chi* genes, the

patterns different from those of wild type DNA fragments. These results show that at least 11 clones of the randomly selected 14 clones contain recombinant DNA reassembled from a pair of the wild-type DNA.

To identify the resulting recombinant DNA, *Hin*dIII/*Xba* I fragment of the 10 plasmids was sequenced using the ABI PRISM Dye terminator Cycle Sequencing Kit (PE Biosystems) and the sequences were compared with those of the wild-type *m-chi* and *l-chi* genes, alignments of which were shown in Fig. 5 and further depicted in the schematic diagram of Fig. 6.

As shown in Fig. 6, recombination between the two wild-type genes took place once as for the recombinant DNA clones 3, 4 and 10; twice as for clones 1, 2, 7 and 8; three times as for clones 6 and 9; and four times as for clone 5. These results suggest that the method of the present invention using unidirectional single-stranded polynucleotides can efficiently generate a random recombinant DNA library from two or more kinds of starting polynucleotides.

To screening a recombinant polynucleotide encoding a chitinase which has specific activity higher than that of wild-type enzyme, the colonies were transferred by replica-plating method to LB-agar plates containing 100μg/ml ampicillin and 0.5% swollen chitin, and incubated at 37℃ overnight until clear plaques were developed. About 800 colonies were screened according to the degree of their clearance. Fig. 7 shows the variance of the sizes of clear zones formed by the colonies expressing the recombinant chitinase depending on their chitin decomposing activities different from each other. A chitinase produced by a colony forming a clear zone larger than wild type was designated R-24 chitinase. Plasmid DNA was extracted from the clone by Qiaprep Spin Miniprep method(Qiagen) and the nucleotide sequence of R-24 chininase gene was analyzed. Fig. 8 compares the nucleotide sequence of R-24 chininase gene (SEQ ID NOs: 13) with those of two wild-type genes, i.e., *l-chi* gene(SEQ ID NO: 1) and *m-chi* gene(SEQ ID NO: 2). Fig. 9 is a schematic diagram showing the constitution of R-24 chininase gene in comparison with the two wild-type genes. From Fig. 9, it can be seen that R-24 chitinase gene was produced by four times of recmbinations between the

two wild-type genes.

Table 1 shows the comparison of the specific activity of R-24 chitinase with those of the two wild-type chitinases.

Table 1: Specific activities of the wild-type chitinases and recombinant R-24 chitinase

Chitinase	Specific activity (U/mg)
Serratia marcescens chitinase	150.6
Serratia liquefaciens chitinase	201.3
R-24 chitinase	227.2

As can be seen from Table 1, specific activity of R-24 chitinase is higher than *Serratia marcescens* chitinase and *Serratia liquefaciens* chitinase by factors of 1.5 and 1.1, respectively.

Example 4 : Directed evolution of a chitosanase for thermostability

4-1) Preparation of mutant chitosanases by error-prone PCR

About 0.5-kb DNA fragment obtained by *Eco*RV/*Sal* I double digestion of pBR322 was inserted into *Eco*RV/*Sal* I digestion site of pBluescript II SK. The resulting vector construct was then cut with *Xba* I and *Eco*R I, and ligated to about 1.4-kb chitosanase gene obtained by digesting *Bacillus sp.* (KCTC 0377BP) with same restriction enzymes, resulting in a recombinant vector construct, pBSK-csn-322, containing chitosanase gene.

The pBSK-csn-322 was used as a template for error-prone polymerase chain reaction. Each 50 pmole of primers csn-*Xba* I (SEQ ID NO: 26) and csn-c1(SEQ ID NO: 27) was used for an error-prone PCR reaction which was performed in 100$\mu\ell$ of PCR mix comprising 10 mM Tris-HCl(pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP 1 mM dCTP, 1 mM

dTTP, 0.15 mM $MnCl_2$, 10 ng of template DNA and 5 units of Taq polymerase using an MJ Research Thermal cycler (PTC-200). The PCR conditions were as follows: 94℃ for 3 min; 94℃ for 30 seconds, 55℃ for 30 seconds and 72℃ for 30 seconds (30 cycles); and followed by 72℃, 5 min.

The resulting 1.4-kb DNA fragment was digested with *Xba* I and *Eco*R I, then ligated to *Xba* I /*Eco*R I backbone of pBSK-csn-322. The resulting recombinant plasmid was transformed to *E. coli* JM83 and positive transformants were selected by culturing them on LB-agar plates supplemented with 100μg/ml ampicillin at 37℃ for 18 hours. The colonies formed on the plates were replica-plated onto a fresh plates and incubated at 37℃ for 20 hours. The petri dish containing the colonies was heated on a water bath at 70℃ for 15 minutes, and then 50 mM Na-acetate buffer solution containing 0.1% chitosan and 1% agarose was poured onto the LB-agar plates. After the plates was placed at 37℃ for 24 hours, colonies still having chitosanase activity to produce clear plaques were selected using 0.2 % Congo Red. As a result of aforementioned process, 9 positive clones having improved thermal stability were isolated out of about 12,000 clones. Plasmid DNAs were extracted from the clones by Qiaprep Spin Miniprep method(Qiagen) and the nucleotide sequences of chitosanase genes therein were analyzed. Table 2 shows the amino acid substitution sites of thermostable mutant chitosanases produced by error-prone PCR in comparison with the wild-type chitosanase.

Table 2: Amino acid substitution sites of thermostable mutant chitosanases produced by error-prone PCR

Mutant chitosanase	Amino acid substitution sites
d10-68	D305G
e3-97	E308G
e4-12	I389M
e15-20	T131I, N368D
e18-5	S24P, T277A, N368D
e22-23	K172E, S376P
e26-27	Q159R
e26-98	E107D, Q442R
e30-97	S376P, Y451C

4-2) Construction of the first recombinant DNA library and screening

DNA reassembly process according to the present invention was carried out using the 9 mutant chitosanase genes selected in 4-1) above as starting polynucleotides.

The plasmids extracted from the 9 clones were mixed in each quantity of 500 ng and then the linearized DNA fragments of about 4.9-kb in size were obtained by *Xho* I digestion. The linearized fragments of 200 ng were transcribed in 20$\mu\ell$ of transcription buffer solution[40 mM Tris-HCl, pH 7.8, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT] containing 0.5 mM each rNTPs, 40 units of RNasin and 17 unist of T3 RNA polymerase at 37℃ for 1 hour. The resulting RNA transcripts of the mutant genes for chitosanase were purified in RNAeasy column(Qiagen).

Reverse Transcription was conducted on 200 ng of the RNA in 50$\mu\ell$ of reaction solution[10 mM Tris-HCl, pH 8.3, 15 mM KCl, 0.6 mM $MgCl_2$, 0.2 mM DTT] containing 6μg of random hexamer, 0.2 mM each dNTPs, 40

units of RNasin and 50 units of M-MLV reverse transcriptase at 37℃ for 1 hour. The template RNA was then removed by RNase I at 37℃ for 1 hour. Through the reverse transcription process, unidirectional single-stranded DNAs of random size were synthesized from the random hexamer annealed with the template RNA. The resulting single-stranded DNA was analyzed by 1% agarose gel electrophoresis and extracted from the gel using a Geneclean kit(Bio 101).

The unidirectional single-stranded DNA fragments obtained above served as templates for polymerase chain reaction. A reaction mixture contained 10 ng of single-stranded DNA fragments, 0.2 mM each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl(pH 8.8), 0.1% Triton X-100, 2 units of Vent DNA polymerase (New England BioLabs) and 25 pmole of csn-*Xba* I primer(SEQ ID NO: 26) in a total volume of 50$\mu\ell$. PCR was carried out on an MJ Research thermal cycler (PTC-100) at 94℃ for 3 min; 94℃ for 30 seconds, 55℃ for 30 seconds, 72℃ for 30 seconds (30 cycles); and 72℃, 5 min. The full-length DNA of about 1.4-kb in size was then amplified by PCR using 25 pmole of csn-c1 primer(SEQ ID NO: 27) under the same conditions as described above. The resulting 1.4-kb DNA was digested with *Xba* I and *Eco*R I and then ligated to the *Xba* I /*Eco*R I backbone of pBSK-csn-322. The resulting plasmid was transformed to *E. coli* KM83 and positive transformants were selected on LB-agar plates supplemented with 100μg/ml ampicillin at 37℃ for 20 hours. Grown colonies were transferred onto fresh plates by replica-plating method and incubated at 37℃ for 20 hours. The plates were heated on water bath at 75℃ for 20 minutes, and then 50 mM Na-acetate solution containing 0.1% chitosan and 1% agarose was added onto the LB-agar plates. After incubated at 37℃ overnight, colonies still having chitosanase activity resulting in clear plaque around them notwithstanding the heat treatment were selected on 0.2% Congo Red.

Through the aforementioned process, 23 clones having improvement in heat resistance compared to the 8 clones obtained by error-prone PCR were selected out of about 12,000 clones.

4-3) Construction of secondary recombinant DNA library and screening

Secondary recombinant DNA library was constructed with the 23 mutant chitosanase genes, which had been obtained by the screening of the first recombinant DNA library, through the same process as described in 4-2) above. After heated at 80℃ for 30 min, the resulting colonies of 16,000 or more were screened for mutant chitosanase having more improved thermal stability than the starting materials, 23 mutant chitosanases. Two mutants were selected and polypeptides encoded by them were designated as M-13 and M-20, respectively.

4-4) Determination of amino acid substitution sites and analysis for thermal stability of M-13 and M-20

From the colonies expressing the mutant chitosanases, M-13 and M-20, plasmid DNAs were extracted and the nucleotide sequences of the chitosanase genes were analyzed. Fig. 10 represents the comparison between the sequences of wild-type chitosanase and the genes encoding M-13 and M-20, respectively. Further, deduced amino acid sequences of the wild-type chitosanase and the thermostable M-13 and M-20 mutants were analyzed and the amino acid substitution sites of the mutant chitosanase different from those of the wild-type chitosanase were presented in Table 3.

Table 3: Amino acid substitution sites of thermostable mutant chitosanases produced by the inventive method

Mutant chitosanase	Amino acid substitution sites
M-13 chitosanase	N60Y, E107D, Q159R, N228T, D305G, E308G, N368D, S376P, F384L, I389M, D435G
M-20 chitosanase	S24P, E107D, Q159R, N286D, D305G, E308G, N357D, N368D, N371D

As can be seen from Table 3, when compared with the substitution sites present in the mutants prepared by the error-prone PCR as shown in Table 2, it

was exhibited that the substitution sites present in seven mutants, i.e., E107D, Q159R, D305G, E308G, N368D, S376P and I389M, were accumulated in M-13 chitosanase; and the substitution sites present in six mutants, i.e., S24P, E107D, Q159R, D305G, E308G and N368D, in M-13 chitosanase, by the recombination. This result demonstrates that the method of the present invention is useful for the efficient production of recombinant polynucleotides. On the other hand, it can be seen that in addition to the substitution sites resulted from the recombination between the parent mutants, new 4 and 3 mutation sites were introduced into M-13 and M-20 mutant chitosanases, respectively, during the process of the inventive method.

In order to determine the thermal stabilities of the mutant chitosanases, the wild-type chitosanase, M-13 mutant and M-20 mutant were treated at 60℃ and the remaining activities according to time were determined. Fig. 11 shows the differences in the thermal stabilities of the wild-type chitosanase, M-13 mutant and M-20 mutant. In Fig. 11, half-lives($T_{1/2}$) of the enzymes, which means that the activity thereof decreases by 50% as compared to the initial activity, are 5.1 min for the wild-type, 6.9 hours for M-13 mutant and 11.6 hours for M-20 mutant. This result shows that the thermal stabilities at 60℃ of M-13 and M-20 mutants increased by 81 and 136 folds, respectively, than the wild-type chitosanase.

As can be appreciated from the disclosure and the examples above, the method of the present invention can be used for *in vitro* recombination of homologous polynucleotides and the directed molecular evolution of proteins for desired properties. It is also contemplated that the method of the present invention has advantages over the conventional methods in that random diversity of the polynucleotides is achieved in a short time.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

What is claimed is:

1. A method for producing recombinant polynucleotides comprising the steps of:

(a) generating a pool of unidirectional single-stranded polynucleotide fragments randomized in length from at least one starting polynucleotide to be reassembled, which have regions of similarity with each other;

(b) conducting a polymerization process comprising multi-cyclic extension reactions, wherein the unidirectional single-stranded polynucleotide fragments prepared by step (a) serve as templates sequentially and specific oligonucleotides are added to the reaction mixture as primers, the primers being extended by means of template switching to produce at least one recombinant polynucleotide, and the resulting recombinant polynucleotides being different from the starting polynucleotides in nucleotide sequence; and

(c) conducting a polymerase chain reaction using at least one specific primer to amplify the recombinant polynucleotides prepared by step (b).

2. The method of claim 1, wherein step (a) comprises:

(i) conducting a transcription process to produce RNA from at least one starting polynucleotide; and

(ii) conducting a reverse transcription process, wherein random primers are used as primers and the RNA transcript of step (i) as a template.

3. The method of claim 1, wherein step (a) comprises:

(i) generating a 3'-overhang on one side of the starting double-stranded polynucleotide by digesting with at least one restriction enzyme;

(ii) producing a pool of double-stranded polynucleotides having unidirectional sequential deletion by treating the reaction mixture of step (i) with exonuclease Ⅲ followed by removing aliquots of the reaction mixture at

a chosen time interval and further blocking the activity of the exonuclease III;

(iii) treating the resulting double-stranded polynucleotides having a 5'-overhang with an S1 nuclease and a DNA polymerase to form a blunt end thereof;

(iv) generating a new 3'-overhang to the same side which has 3'-overhang in step (i); and

(v) treating the polynucleotides of step (iv) with exonuclease III to generate single-stranded polynucleotide fragments.

4. The method of claim 1, wherein step (a) comprises:

(i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme;

(ii) treating the polynucleotides of step (i) with exonuclease III to generate single-stranded polynucleotides; and

(iii) conducting a polymerization process on the single-stranded polynucleotides of step (ii) using random primers.

5. The method of claim 1, wherein step (a) comprises:

(i) generating a 3'-overhang on one side of the starting double-stranded polynucleotides by digesting with at least one restriction enzyme;

(ii) treating the polynucleotides of step (i) with exonuclease III to generate single-stranded polynucleotides; and

(iii) producing a pool of single-stranded polynucleotides having unidirectional sequential deletion by treating the single-stranded polynucleotides of step (ii) with a single-strand specific 5'→3' exonuclease followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease.

6. The method of claim 1, wherein step (a) comprises:

(i) conducting a polymerase chain reaction on the starting double-stranded polynucleotides using only one kind of oligonucleotide among forward and reverse primers;

(ii) isolating the resulting single-stranded polynucleotides from the starting double-stranded polynucleotides; and

(iii) conducting a polymerization process on the single-stranded polynucleotides of step (ii) using random primers.

7. The method of claim 1, wherein step (a) comprises:

(i) conducting a polymerase chain reaction on the starting double-stranded polynucleotides using only one kind of oligonucleotide among forward and reverse primers;

(ii) isolating the resulting single-stranded polynucleotides from the starting double-stranded polynucleotides; and

(iii) producing a pool of single-stranded polynucleotides having unidirectional sequential deletion by treating the single-stranded polynucleotides of step (ii) with a single-strand specific 5'→3' exonuclease followed by removing aliquots of the reaction mixture at a chosen time interval and further blocking the activity of the exonuclease.

8. The method of claim 1, wherein step (a) comprises:

(i) isolating a single-stranded polynucleotide from a viral vector or plasmid vector which has at least one starting polynucleotide insert; and

(ii) conducting a polymerization process on the single-stranded polynucleotides of step (i) using random primers.

9. The method of claim 1, wherein step (b) comprises the steps of:

(i) conducting at least one cycle wherein the primers are extended to the end of the unidirectional single-stranded DNA fragments used as templates;

(ii) conducting at least one subsequent cycle wherein each of the resulting extended polynucleotides of step (i) is further extended to the end of an unidirectional single-stranded DNA fragment other than the unidirectional single-stranded DNA fragment used in step (i) by means of template switching; and

(iii) repeating step (ii) until recombinant polynucleotides of desired length are obtained.

10. The method of claim 1, wherein the specific oligonucleotides of step (b) have specific nucleotide sequences which is capable of hybridizing with at least one starting polynucleotide.

11. The method of claim 1, wherein the starting polynucleotide is a gene encoding any one of proteins selected from the group consisting of enzymes, antibodies, antigens, binding proteins, hormones, growth factors and plasma proteins, or a part thereof.

12. The method of claim 11, wherein the enzyme is selected from the group consisting of hydrolase, lyase, transferase, oxidoreductase, ligase and isomerase.

13. The method of claim 1, wherein the starting polynucleotide is a wild type DNA or a mutant type DNA obtained therefrom.

14. A method for constructing a recombinant DNA library, comprising the steps of inserting the recombinant polynucleotide prepared by the method of any one of claims 1 to 10 into a vector; and transforming an expression cell with said vector containing the recombinant polynucleotide to obtain a

plurality of mutant clones.

15. The method of claim 14, wherein the vector is selected from the group consisting of a phage, a plasmid, a phagemid, a viral vector and an artificial chromosome.

16. The method of claim 14, wherein the expression cell is selected from the group consisting of bacteria, fungi, plant cells, animal cells and insect cells.

17. A method for evolving a polynucleotide toward a desired property which comprises screening recombinant polynucleotides having a desired functional properties from the recombinant DNA library constructed by the method of claim 14.

FIG. 1

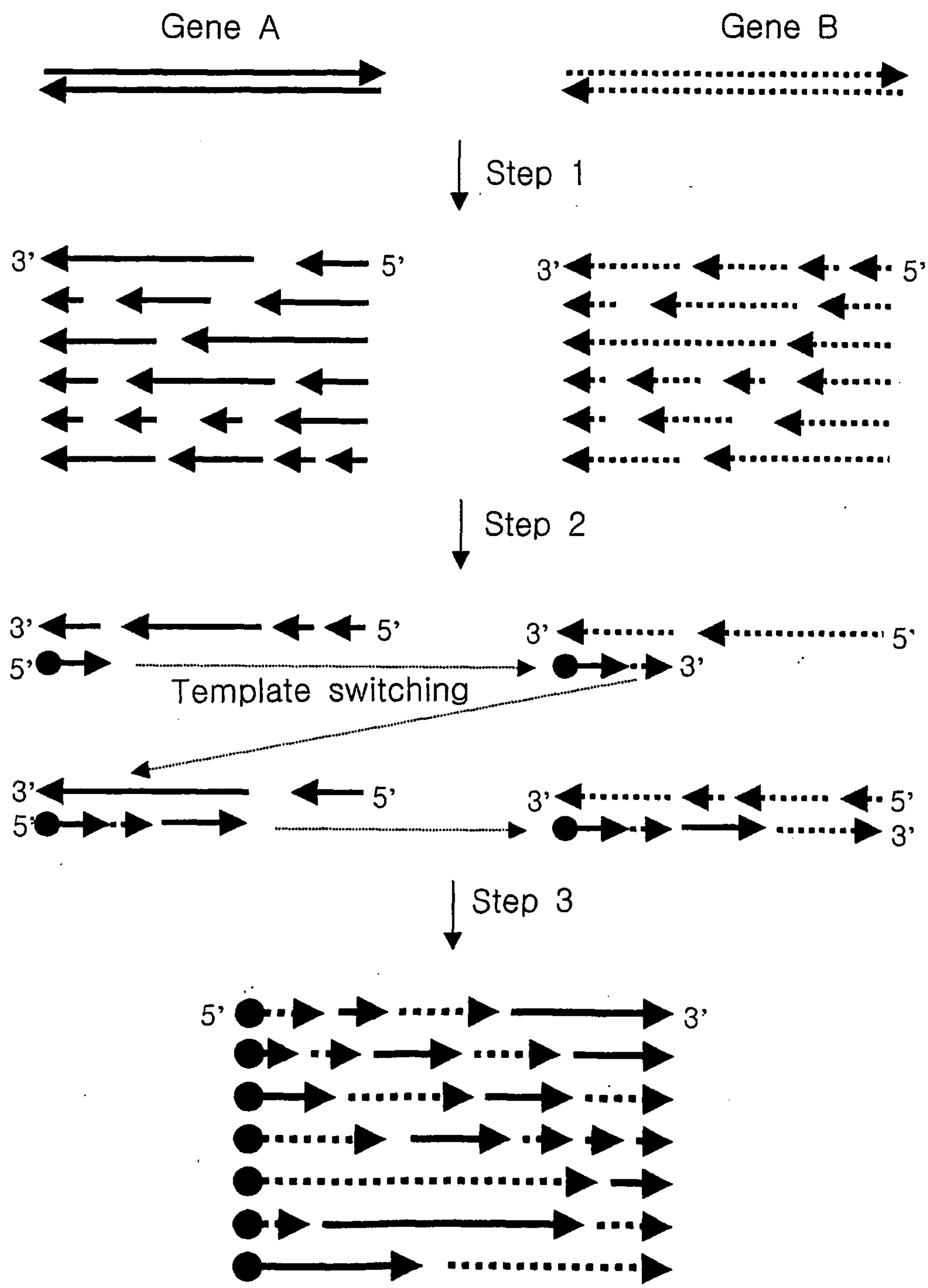
Gene A
Gene B
Step 1
3'
5'
3'
5'
Step 2
3'
5'
5'
3'
5'
3'
Template switching
3'
5'
5'
3'
5'
3'
Step 3
5'
3'

FIG. 2

```
l-chi  ATGCGCAAAT TTAATAAACC GCTGTTGGCG tTGcTGATCG GCAGCACGCT   50
m-chi  ATGCGCAAAT TTAATAAACC GCTGTTGGCG cTGtTGATCG GCAGCACGCT   50

l-chi  GTGcTCtGCG GCGCAGGCCG CtGCaCCGGG CAAaCCtACg tTgGCCTGGG  100
m-chi  GTGtTCcGCG GCGCAGGCCG CcGCgCCGGG CAAgCCgACc aTcGCCTGGG  100

l-chi  GCAAtACCAA aTTCGCCATt GTcGAAGTcG AtCAaGCGGC gACgGCTTAT  150
m-chi  GCAAcACCAA gTTCGCCATc GTtGAAGTtG AcCAgGCGGC tACcGCTTAT  150

l-chi  AATAATcTGG TGAAaGTAAA AAgTGCCGCC GAcGTTTCtG TtTCaTGGAA  200
m-chi  AATAATtTGG TGAAgGTAAA AAaTGCCGCC GAtGTTTCcG TcTCcTGGAA  200

l-chi  TTTATGGAAT GGCGAtaCcG GtACcACGGC aAAagTaTTA TTAAATGGcA  250
m-chi  TTTATGGAAT GGCGAcgCgG GcACgACGGC cAAgaTtTTA TTAAATGGtA  250

l-chi  AAGAaGttTG GAGTGGTgCc TCAACCGGta gTTCgGGaAC cGCaAAcTTT  300
m-chi  AAGAgGcgTG GAGTGGTcCt TCAACCGGat cTTCcGGtAC gGCgAAtTTT  300

l-chi  AAggtgaATA AAGGCGGCCG TTATCAAATG CAGGTGGCgT TaTGCAAcGC  350
m-chi  AAaagtgATA AAGGCGGCCG TTATCAAATG CAGGTGGcaT TgTGCAAtGC  350

l-chi  CGACGGCTGt ACCGCCAGcG AtGCaACCGA AATTGTGGTG GCaGAtACCG  400
m-chi  CGACGGCTGc ACCGCCAGtG AcGCcACCGA AATTGTGGTG GCcGAcACCG  400

l-chi  ACGGtAGCCA TTTGGCaCCt TTaAAAGAaC CttTGtTGGA AAAGAATAAg  450
m-chi  ACGGcAGCCA TTTGGCgCCg TTgAAAGAgC CgcTGcTGGA AAAGAATAAa  450

l-chi  CCtTATAAAC AagACTCCGG CAAAGTGGTt GGcTCTTATT TCGTtGAaTG  500
m-chi  CCgTATAAAC AgaACTCCGG CAAAGTGGTc GGtTCTTATT TCGTcGAgTG  500

l-chi  GGGCGTTTAC GGcCGtAATT TCACCGTCGA tAAacTtCCG GCtCAgAACC  550
m-chi  GGGCGTTTAC GGgCGcAATT TCACCGTCGA cAAgaTcCCG GCgCAaAACC  550

l-chi  TGACgCACCT GCTGTACGGC TTTATCCCtA TCTGtGGCGG tgAcGGCATC  600
m-chi  TGACcCACCT GCTGTACGGC TTTATCCCgA TCTGcGGCGG caAtGGCATC  600

l-chi  AACGACAGCC TGAAAGAGAT cGAAGGCAGC TTCCAGGCGT TACAGCGtTC  650
m-chi  AACGACAGCC TGAAAGAGAT tGAAGGCAGC TTCCAGGCGT TACAGCGcTC  650

l-chi  CTGtCAGGGg CGtGAaGACT TtAAgGTaTC GaTCCACGAT CCGTTCGCtG  700
m-chi  CTGcCAGGGc CGcGAgGACT TcAAaGTcTC GgTCCACGAT CCGTTCGCcG  700

l-chi  CGCTGCAgAA AGgtCAGAAG GGCGTGACCG CCTGGGAcGA CCCCTACAAa  750
m-chi  CGCTGCAaAA AGcgCAGAAG GGCGTGACCG CCTGGGAtGA CCCCTACAAg  750

l-chi  GGCAACTTCG GCCAGtTGAT GGCGtTGAAa CAGGCGCgcC CgGACCTGAA  800
m-chi  GGCAACTTCG GCCAGcTGAT GGCGcTGAAg CAGGCGCatC CtGACCTGAA  800

l-chi  AATCCTGCCG TCGATCGGtG GCTGGACGtT aTCCGAtCCG TTCTTCTTtA  850
m-chi  AATCCTGCCG TCGATCGGcG GCTGGACGcT gTCCGAcCCG TTCTTCTTcA  850
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FIG. 2 (continued)

```
l-chi  TGGGCGAtAA GGTGAAGCGC GATCGCTTCG TCGGcTCGGT GAAgGAGTTC   900
m-chi  TGGGCGAcAA GGTGAAGCGC GATCGCTTCG TCGGtTCGGT GAAaGAGTTC   900

l-chi  CTGCAaACCT GGAAGTTCTT tGAtGGCGTa GATATCGACT GGGAaTTCCC   950
m-chi  CTGCAgACCT GGAAGTTCTT cGAcGGCGTg GATATCGACT GGGAgTTCCC   950

l-chi  GGGCGGgcAg GGtGCtAACC CgAAaCTGGG CAGtaCGCAg GAtGGGGcAA   1000
m-chi  GGGCGGcaAa GGcGCcAACC CtAAcCTGGG CAGccCGCAa GAcGGGGaAA   1000

l-chi  CCTATGTGca GCTGATGAAa GAGCTGCGcG CcATGCTGGA TCAGCTtTCG   1050
m-chi  CCTATGTGtt GCTGATGAAg GAGCTGCGgG CgATGCTGGA TCAGCTgTCG   1050

l-chi  GCGGAAACgG GCCGtAAGTA TGAaCTGACC TCtGCgATCA GCGCCGGcAA   1100
m-chi  GCGGAAACcG GCCGcAAGTA TGAgCTGACC TCcGCcATCA GCGCCGGtAA   1100

l-chi  GGAtAAaATC GAtAAGGTGG aTTACAACac cGCaCAaAAC TCGATGGATC   1150
m-chi  GGAcAAgATC GAcAAGGTGG cTTACAACgt tGCgCAgAAC TCGATGGATC   1150

l-chi  ACATtTTCCT GATGAGtTAC GACTTCTATG GgGCaTTCGA TCTGAAaAAt   1200
m-chi  ACATcTTCCT GATGAGcTAC GACTTCTATG GcGCcTTCGA TCTGAAgAAc   1200

l-chi  CTGGGcCAcC AGACtGCGCT GAAaGCGCCG GCCTGGAAaC CGGAtACgGC   1250
m-chi  CTGGGgCAtC AGACcGCGCT GAAtGCGCCG GCCTGGAAgC CGGAcACcGC   1250

l-chi  gTAtACCACG GTGAAtGGCG TtAATGCaCT GCTcaCGCAG GGCGTgAAGC   1300
m-chi  tTAcACCACG GTGAAcGGCG TcAATGCgCT GCTggCGCAG GGCGTcAAGC   1300

l-chi  CGGGCAAaAT CGTGGTgGGC ACCGCCATGT AcGGtCGCGG tTGGACCGGG   1350
m-chi  CGGGCAAgAT CGTGGTcGGC ACCGCCATGT AtGGcCGCGG cTGGACCGGG   1350

l-chi  GTGAACGGtT ACCAGAACAA CATTCCGTTt ACCGGcACCG CCACTGGcCC   1400
m-chi  GTGAACGGcT ACCAGAACAA CATTCCGTTc ACCGGtACCG CCACTGGgCC   1400

l-chi  GGTgAAAGGC ACCTGGGAaA AtGGCATCGT GGAtTACCGC CAgATCGCCa   1450
m-chi  GGTtAAAGGC ACCTGGGAgA AcGGCATCGT GGAcTACCGC CAaATCGCCg   1450

l-chi  atgAGTTtAT GAGCGGCGAa TGGCAGTAcA gCTACGAtGC tACcGCtGAA   1500
m-chi  gccAGTTcAT GAGCGGCGAg TGGCAGTAtA cCTACGAcGC cACgGCgGAA   1500

l-chi  GCaCCcTAtG TcTTCAAACC TTCCACtGGC GATCTGATCA CCTTCGACGA   1550
m-chi  GCgCCtTAcG TgTTCAAACC TTCCACcGGC GATCTGATCA CCTTCGACGA   1550

l-chi  TGCgCGCTCG GTGCAGGCgA AgGGCAAaTA tGTGCTGGAT AAGCAGCTGG   1600
m-chi  TGCcCGCTCG GTGCAGGCcA AaGGCAAgTA cGTGCTGGAT AAGCAGCTGG   1600

l-chi  GCGGgtTGTT CTCaTGGGAa ATtGACGCcG AcAACGGCGA TATTCTgAAt   1650
m-chi  GCGGccTGTT CTCcTGGGAg ATcGACGCgG AtAACGGCGA TATTCTcAAc   1650

l-chi  AaCATGAACa gCAGCCTGGG CAACAGCGtC GGtacgCctT AA           1692
m-chi  AgCATGAACg cCAGCCTGGG CAACAGCGcC GGcgttCaaT AA           1692
```

FIG. 3

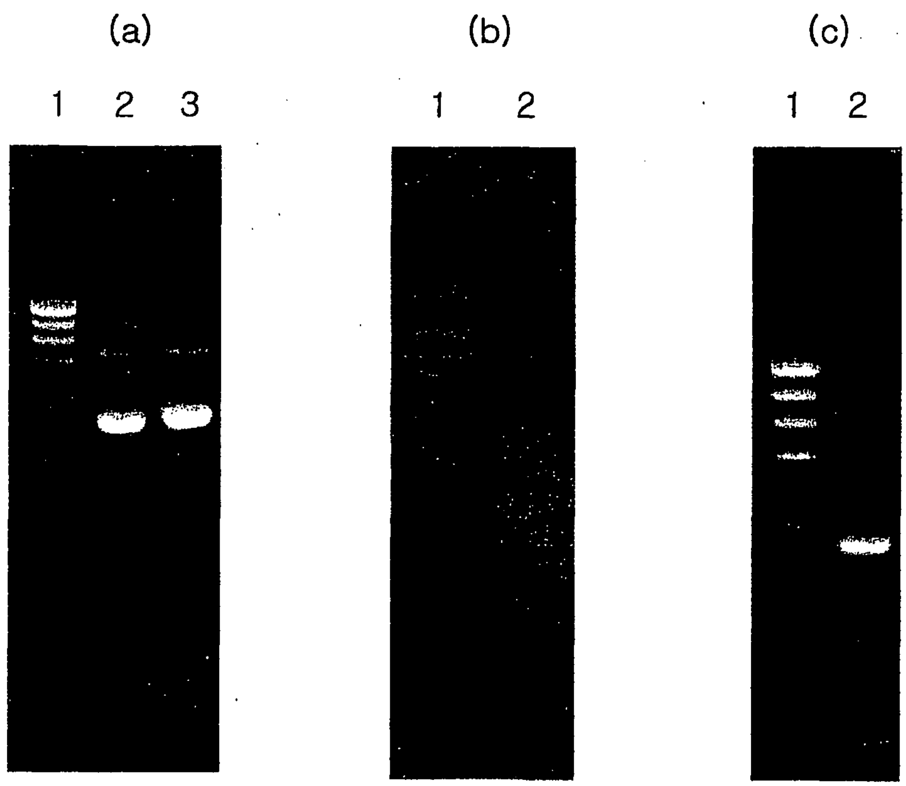

FIG. 4

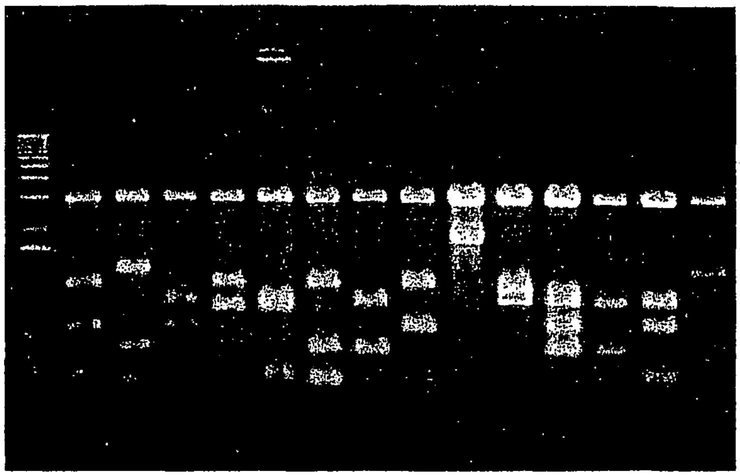

FIG. 5

l-chi	ATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCG	60
m-chi	ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG	60
mut-1	ATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCG	60
mut-2	ATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCG	60
mut-3	ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG	60
mut-4	ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG	60
mut-5	ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG	60
mut-6	ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG	60
mut-7	ATGCGCAAATTTAATAAACCGCTGTTGGCGTTGCTGATCGGCAGCACGCTGTGCTCTGCG	60
mut-8	ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG	60
mut-9	ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG	60
mut-10	ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG	60

l-chi	GCGCAGGCCGCTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATT	120
m-chi	GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC	120
mut-1	GCGCAGGCCGCTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATT	120
mut-2	GCGCAGGCCGCTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATT	120
mut-3	GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC	120
mut-4	GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC	120
mut-5	GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC	120
mut-6	GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC	120
mut-7	GCGCAGGCCGCTGCACCGGGCAAACCTACGTTGGCCTGGGGCAATACCAAATTCGCCATT	120
mut-8	GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC	120
mut-9	GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC	120
mut-10	GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAACACCAAGTTCGCCATC	120

l-chi	GTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC	180
m-chi	GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC	180
mut-1	GTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC	180
mut-2	GTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC	180
mut-3	GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC	180
mut-4	GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC	180
mut-5	GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC	180
mut-6	GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC	180
mut-7	GTCGAAGTCGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC	180
mut-8	GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC	180
mut-9	GTTGAAGTTGATCAAGCGGCGACGGCTTATAATAATCTGGTGAAAGTAAAAAGTGCCGCC	180
mut-10	GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAATTTGGTGAAGGTAAAAAATGCCGCC	180

FIG. 5 (continued)

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l-chi    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
m-chi    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-1    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
mut-2    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
mut-3    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-4    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-5    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-6    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-7    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
mut-8    GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240
mut-9    GACGTTTCTGTTTCATGGAATTTATGGAATGGCGATACCGGTACCACGGCAAAAGTATTA 240
mut-10   GATGTTTCCGTCTCCTGGAATTTATGGAATGGCGACGCGGGCACGACGGCCAAGATTTTA 240

l-chi    TTAAATGGCAAAGAAGTTTGGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
m-chi    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-1    TTAAATGGCAAAGAAGTTTGGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
mut-2    TTAAATGGCAAAGAAGTTTGGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
mut-3    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-4    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-5    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-6    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-7    TTAAATGGCAAAGAAGTTTGGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
mut-8    TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300
mut-9    TTAAATGGCAAAGAAGTTTAGAGTGGTGCCTCAACCGGTAGTTCGGGAACCGCAAACTTT 300
mut-10   TTAAATGGTAAAGAGGCGTGGAGTGGTCCTTCAACCGGATCTTCCGGTACGGCGAATTTT 300

l-chi    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
m-chi    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-1    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-2    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-3    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-4    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-5    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-6    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
mut-7    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-8    AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-9    AAGGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCGTTATGCAACGCCGACGGCTGT 360
mut-10   AAAGTGAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
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FIG. 5 (continued)

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l-chi    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
m-chi    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-1    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-2    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-3    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-4    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-5    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-6    ACCGCCAGTGACGCCACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-7    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-8    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
mut-9    ACCGCCAGCGATGCAACCGAAATTGTGGTGGCAGATACCGACGGTAGCCATTTGGCACCT 420
mut-10   ACCGCCAGTGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420

l-chi    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
m-chi    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-1    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTC 480
mut-2    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
mut-3    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-4    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-5    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-6    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
mut-7    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
mut-8    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480
mut-9    TTAAAAGAACCTTTGTTGGAAAAGAATAAGCCTTATAAACAAGACTCCGGCAAAGTGGTT 480
mut-10   TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTGGTC 480

l-chi    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
m-chi    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-1    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-2    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-3    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-4    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-5    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-6    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-7    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-8    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
mut-9    GGCTCTTATTTCGTTGAATGGGGCGTTTACGGCCGTAATTTCACCGTCGATAAACTTCCG 540
mut-10   GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
```

FIG. 5 (continued)

```
l-chi     GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
m-chi     GCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAATGGCATC 600
mut-1     GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-2     GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-3     GCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAATGGCATC 600
mut-4     GCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-5     GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-6     GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-7     GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-8     GCGCAAAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAATGGCATC 600
mut-9     GCTCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCTATCTGTGGCGGTGACGGCATC 600
mut-10    GCGCAGAACCTGACGCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGTGATGGCATC 600

l-chi     AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
m-chi     AACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGCCAGGGC 660
mut-1     AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-2     AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-3     AACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGCCAGGGC 660
mut-4     AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-5     AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-6     AACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-7     AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-8     AACGACAGCCTGAAAGAGATTGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGCCAGGGC 660
mut-9     AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGTTCCTGTCAGGGG 660
mut-10    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGTTACAGCGCTCCTGTCAGGGG 660

l-chi     CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
m-chi     CGCGAGGACTTCAAAGTCTCGGTCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
mut-1     CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-2     CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-3     CGCGAGGACTTCAAAGTCTCGGTCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
mut-4     CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-5     CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-6     CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCAGAAAGGTCAGAAG 720
mut-7     CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
mut-8     CGCGAGGACTTCAAAGTCTCGGTCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
mut-9     CGTGAAGACTTTAAGGTATCGATCCACGATCCGTTCGCTGCGCTGCCGAAAGGTCAGAAG 720
mut-10    CGCGAAGACTTCAAGGTATCGGTCCACGATCCGTTCGCCGCGCTGCAGAAAGGGCAGAAG 720
```

FIG. 5(continued)

```
l-chi    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
m-chi    GGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780
mut-1    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-2    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-3    GGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780
mut-4    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-5    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-6    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-7    GGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780
mut-8    GGCGTGACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780
mut-9    GGCGTGACCGCCTGGGACGACCCCTACAAAGGCAACTTCGGCCAGTTGATGGCGTTGAAA 780
mut-10   GGCGTGACCGCCTGGGACGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAG 780

l-chi    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
m-chi    CAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG 840
mut-1    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-2    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-3    CAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGTTATCCGATCCG 840
mut-4    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-5    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-6    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-7    CAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG 840
mut-8    CAGGCGCATCCTGACCTGAAAATCCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG 840
mut-9    CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840
mut-10   CAGGCGCGCCCGGACCTGAAAATCCTGCCGTCGATCGGTGGCTGGACGTTATCCGATCCG 840

l-chi    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
m-chi    TTCTTCTTCATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900
mut-1    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
mut-2    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAAGAGTTC 900
mut-3    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
mut-4    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
mut-5    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAAGAGTTC 900
mut-6    TTCTTCTTTATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900
mut-7    TTCTTCTTCATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900
mut-8    TTCTTCTTAATGGGCGACAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900
mut-9    TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
mut-10   TTCTTCTTTATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGCTCGGTGAAGGAGTTC 900
```

FIG. 5(continued)

```
l-chi   CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
m-chi   CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-1   CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
mut-2   CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-3   CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
mut-4   CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
mut-5   CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-6   CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-7   CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCCAA 960
mut-8   CTGCAGACCTGGAAGTTCTTCGACGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 960
mut-9   CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960
mut-10  CTGCAAACCTGGAAGTTCTTTGATGGCGTAGATATCGACTGGGAATTCCCGGGCGGGCAG 960

l-chi   GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
m-chi   GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-1   GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
mut-2   GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-3   GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
mut-4   GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
mut-5   GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-6   GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-7   GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-8   GGCGCCAACCCTAACCTGGGCAGCCCGCAAGACGGGGAAACCTATGTGTTGCTGATGAAG 1020
mut-9   GGTGCTAACCCGAAACTGGGCAGTACGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020
mut-10  GGTGCTAACCCGAAACTGGGCAGTATGCAGGATGGGGCAACCTATGTGCAGCTGATGAAA 1020

l-chi   GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
m-chi   GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-1   GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
mut-2   GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-3   GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
mut-4   GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
mut-5   GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-6   GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-7   GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-8   GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAGTATGAGCTGACC 1080
mut-9   GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
mut-10  GAGCTGCGCGCCATGCTGGATCAGCTTTCGGCGGAAACGGGCCGTAAGTATGAACTGACC 1080
```

FIG. 5 (continued)

l-chi	TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC	1140
m-chi	TCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAAC	1140
mut-1	TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC	1140
mut-2	TCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAAC	1140
mut-3	TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC	1140
mut-4	TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC	1140
mut-5	TCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAAC	1140
mut-6	TCCGCCATCAGCGCCGGTAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC	1140
mut-7	TCCGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC	1140
mut-8	TCCGCCATCAGCGCCGGTAAGGACAAGATCGACAAGGTGGCTTACAACGTTGCGCAGAAC	1140
mut-9	TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC	1140
mut-10	TCTGCGATCAGCGCCGGCAAGGATAAAATCGATAAGGTGGATTACAACACCGCACAAAAC	1140

l-chi	TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT	1200
m-chi	TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC	1200
mut-1	TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT	1200
mut-2	TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC	1200
mut-3	TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT	1200
mut-4	TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT	1200
mut-5	TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC	1200
mut-6	TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT	1200
mut-7	TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT	1200
mut-8	TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC	1200
mut-9	TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCCTTCGATCTGAAGAAC	1200
mut-10	TCGATGGATCACATTTTCCTGATGAGTTACGACTTCTATGGGGCATTCGATCTGAAAAAT	1200

l-chi	CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG	1260
m-chi	CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG	1260
mut-1	CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG	1260
mut-2	CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG	1260
mut-3	CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG	1260
mut-4	CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG	1260
mut-5	CTGGGGCATCAGACCGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG	1260
mut-6	CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG	1260
mut-7	CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG	1260
mut-8	CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG	1260
mut-9	CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG	1260
mut-10	CTGGGCCACCAGACTGCGCTGAAAGCGCCGGCCTGGAAACCGGATACGGCGTATACCACG	1260

FIG. 5 (continued)

l-chi	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
m-chi	GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAGATCGTGGTCGGC	1320
mut-1	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-2	GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAGATCGTGGTCGGC	1320
mut-3	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-4	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-5	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-6	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-7	GTGAATGGCGTTAATGCACTGCTCGCGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-8	GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAGATCGTGGTCGGC	1320
mut-9	GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAAATCGTGGTGGGC	1320
mut-10	GTGAATGGCGTTAATGCACTGCTCACGCAGGGCGTGAAGCCGGGCAAAATCGTGGTGGGC	1320

l-chi	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
m-chi	ACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCGTTC	1380
mut-1	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-2	ACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCGTTC	1380
mut-3	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-4	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-5	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-6	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-7	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-8	ACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCGTTC	1380
mut-9	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380
mut-10	ACCGCCATGTACGGTCGCGGTTGGACCGGGGTGAACGGTTACCAGAACAACATTCCGTTT	1380

l-chi	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
m-chi	ACCGGTACCGCCACTGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGC	1440
mut-1	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-2	ACCGGTACCGCCACTGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGC	1440
mut-3	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-4	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-5	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-6	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-7	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-8	ACCGGTACCGCCACTGGGCCGGTTAAAGGCACCTGGGAGAACGGCATCGTGGACTACCGC	1440
mut-9	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440
mut-10	ACCGGCACCGCCACTGGCCCGGTGAAAGGCACCTGGGAAAATGGCATCGTGGATTACCGC	1440

FIG. 5(continued)

```
l-chi    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
m-chi    CAAATCGCCGGCCAGTTCATGAGCGGCGAGTGGCAGTATACCTACGACGCCACGGCGGAA 1500
mut-1    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
mut-2    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
mut-3    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
mut-4    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
mut-5    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
mut-6    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
mut-7    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
mut-8    CAAATCGCCGGCCAGTTCATGAGCGGCGAGTGGCAGTATACCTACGACGCCACGGCGGAA 1500
mut-9    CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500
mut-10   CAGATCGCCAATGAGTTTATGAGCGGCGAATGGCAGTACAGCTACGATGCTACCGCTGAA 1500

l-chi    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560
m-chi    GCGCCTTACGTGTTCAAACCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGCTCG 1560
mut-1    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560
mut-2    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560
mut-3    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560
mut-4    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560
mut-5    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCCCGCTCG 1560
mut-5    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCCCGCTCG 1560
mut-6    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560
mut-7    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560
mut-8    GCGCCTTACGTGTTCAAACCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGCTCG 1560
mut-9    GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560
mut-10   GCACCCTATGTCTTCAAACCTTCCACTGGCGATCTGATCACCTTCGACGATGCGCGCTCG 1560

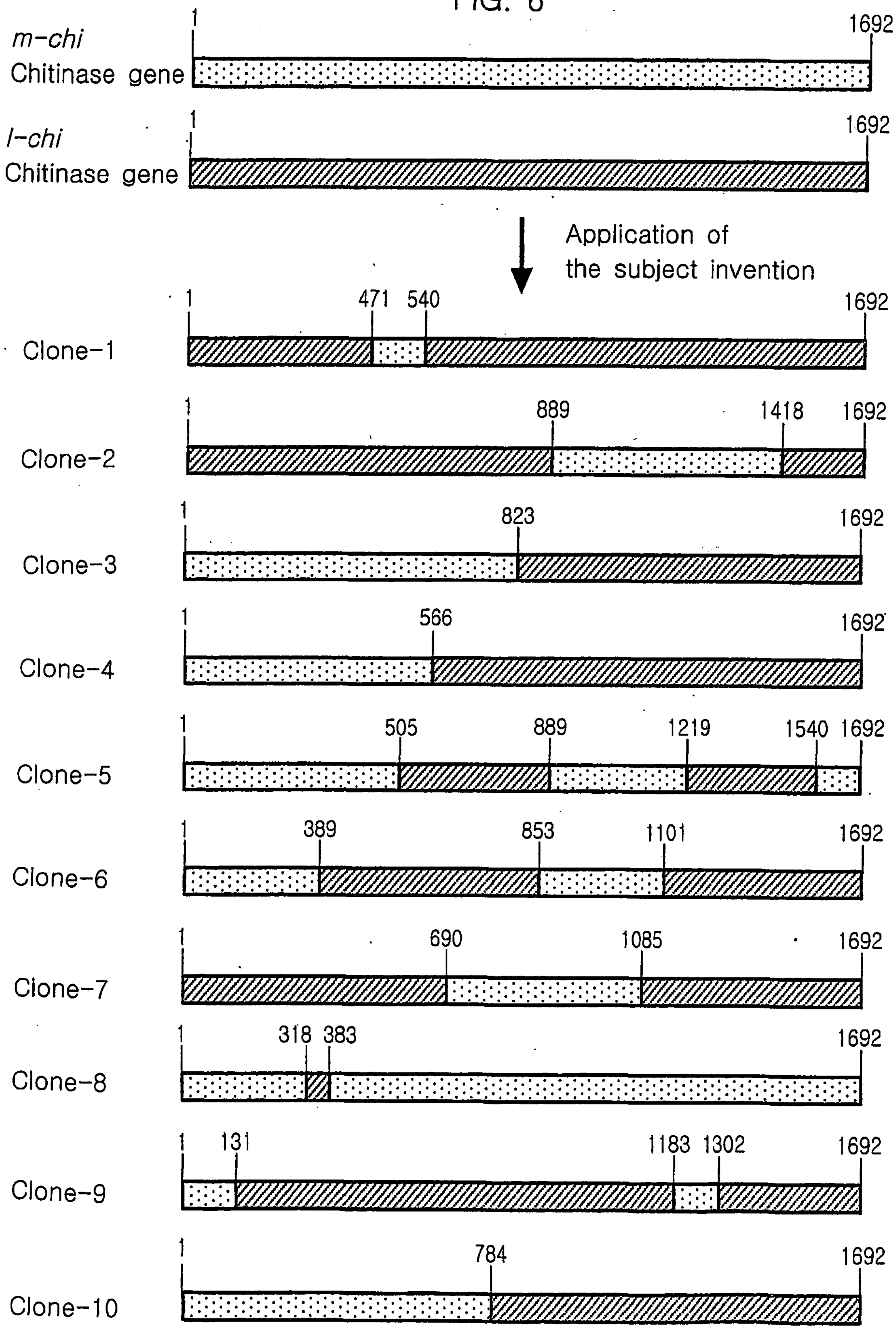
l-chi    GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
m-chi    GTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCCTGGGAG 1620
mut-1    GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
mut-2    GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
mut-3    GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
mut-4    GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
mut-5    GTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCCTGGGAG 1620
mut-6    GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
mut-7    GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
mut-8    GTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCCTGGGAG 1620
mut-9    GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
mut-10   GTGCAGGCGAAGGGCAAATATGTGCTGGATAAGCAGCTGGGCGGGTTGTTCTCATGGGAA 1620
```

FIG. 5 (continued)

```
l-chi    ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680
m-chi    ATCGACGCGGATAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAACAGCGCC 1680
mut-1    ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680
mut-2    ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680
mut-3    ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680
mut-4    ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680
mut-5    ATCGACGCGGATAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAACAGCGCC 1680
mut-6    ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680
mut-7    ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680
mut-8    ATCGACGCGGATAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAACAGCGCC 1680
mut-9    ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680
mut-10   ATTGACGCCGACAACGGCGATATTCTGAATAACATGAACAGCAGCCTGGGCAACAGCGTC 1680

l-chi    GGTACGCCTTAA 1692
m-chi    GGCGTTCAATAA 1692
mut-1    GGTACGCCTTAA 1692
mut-2    GGTACGCCTTAA 1692
mut-3    GGTACGCCTTAA 1692
mut-4    GGTACGCCTTAA 1692
mut-5    GGCGTTCAATAA 1692
mut-6    GGTACGCCTTAA 1692
mut-7    GGTACGCCTTAA 1692
mut-8    GGCGTTCAATAA 1692
mut-9    GGTACGCCTTAA 1692
mut-10   GGTACGCCTTAA 1692
```

FIG. 6


m-chi
Chitinase gene
1
1692
l-chi
Chitinase gene
1
1692
Application of
the subject invention
Clone-1
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471
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1692
Clone-2
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889
1418
1692
Clone-3
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823
1692
Clone-4
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1
505
889
1219
1540
1692
Clone-6
1
389
853
1101
1692
Clone-7
1
690
1085
1692
Clone-8
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318
383
1692
Clone-9
1
131
1183
1302
1692
Clone-10
1
784
1692

FIG. 7

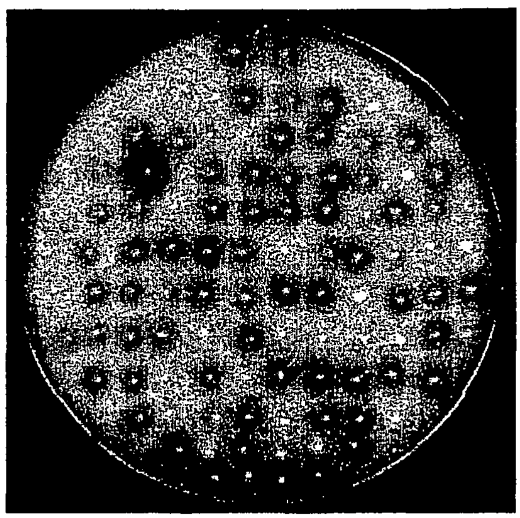


FIG. 8

l-chi	ATGCGCAAAT	TTAATAAACC	GCTGTTGGCG	TTGCTGATCG	GCAGCACGCT	50
m-chi	ATGCGCAAAT	TTAATAAACC	GCTGTTGGCG	CTGTTGATCG	GCAGCACGCT	50
R-24	ATGCGCAAAT	TTAATAAACC	GCTGTTGGCG	TTGCTGATCG	GCAGCACGCT	50
l-chi	GTGCTCTGCG	GCGCAGGCCG	CTGCACCGGG	CAAACCTACG	TTGGCCTGGG	100
m-chi	GTGTTCCGCG	GCGCAGGCCG	CCGCGCCGGG	CAAGCCGACC	ATCGCCTGGG	100
R-24	GTGCTCTGCG	GCGCAGGCCG	CTGCACCGGG	CAAACCTACG	TTGGCCTGGG	100
l-chi	GCAATACCAA	ATTCGCCATT	GTCGAAGTCG	ATCAAGCGGC	GACGGCTTAT	150
m-chi	GCAACACCAA	GTTCGCCATC	GTTGAAGTTG	ACCAGGCGGC	TACCGCTTAT	150
R-24	GCAATACCAA	ATTCGCCATT	GTCGAAGTCG	ATCAAGCGGC	GACGGCTTAT	150
l-chi	AATAATCTGG	TGAAAGTAAA	AAGTGCCGCC	GACGTTTCTG	TTTCATGGAA	200
m-chi	AATAATTTGG	TGAAGGTAAA	AAATGCCGCC	GATGTTTCCG	TCTCCTGGAA	200
R-24	AATAATCTGG	TGAAAGTAAA	AAGTGCCGCC	GACGTTTCTG	TTTCATGGAA	200
l-chi	TTTATGGAAT	GGCGATACCG	GTACCACGGC	AAAAGTATTA	TTAAATGGCA	250
m-chi	TTTATGGAAT	GGCGACGCGG	GCACGACGGC	CAAGATTTTA	TTAAATGGTA	250
R-24	TTTATGGAAT	GGCGATACCG	GTACCACGGC	AAAAGTATTA	TTAAATGGCA	250
l-chi	AAGAAGTTTG	GAGTGGTGCC	TCAACCGGTA	GTTCGGGAAC	CGCAAACTTT	300
m-chi	AAGAGGCGTG	GAGTGGTCCT	TCAACCGGAT	CTTCCGGTAC	GGCGAATTTT	300
R-24	AAGAAGTTTG	GAGTGGTGCC	TCAACCGGTA	GTTCGGGAAC	CGCAAACTTT	300
l-chi	AAGGTGAATA	AAGGCGGCCG	TTATCAAATG	CAGGTGGCGT	TATGCAACGC	350
m-chi	AAAGTGAATA	AAGGCGGCCG	TTATCAAATG	CAGGTGGCAT	TGTGCAATGC	350
R-24	AAGGTGAATA	AAGGCGGCCG	TTATCAAATG	CAGGTGGCGT	TATGCAACGC	350
l-chi	CGACGGCTGT	ACCGCCAGCG	ATGCAACCGA	AATTGTGGTG	GCAGATACCG	400
m-chi	CGACGGCTGC	ACCGCCAGTG	ACGCCACCGA	AATTGTGGTG	GCCGACACCG	400
R-24	CGACGGCTGT	ACCGCCAGCG	ATGCAACCGA	AATTGTGGTG	GCAGATACCG	400
l-chi	ACGGTAGCCA	TTTGGCACCT	TTAAAAGAAC	CTTTGTTGGA	AAAGAATAAG	450
m-chi	ACGGCAGCCA	TTTGGCGCCG	TTGAAAGAGC	CGCTGCTGGA	AAAGAATAAA	450
R-24	ACGGTAGCCA	TTTGGCACCT	TTAAAAGAAC	CTTTGTTGGA	AAAGAATAAG	450
l-chi	CCTTATAAAC	AAGACTCCGG	CAAAGTGGTT	GGCTCTTATT	TCGTTGAATG	500
m-chi	CCGTATAAAC	AGAACTCCGG	CAAAGTGGTC	GGTTCTTATT	TCGTCGAGTG	500
R-24	CCTTATAAAC	AAGACTCCGG	CAAAGTGGTC	GGTTCTTATT	TCGTCGAGTG	500
l-chi	GGGCGTTTAC	GGCCGTAATT	TCACCGTCGA	TAAACTTCCG	GCTCAGAACC	550
m-chi	GGGCGTTTAC	GGGCGCAATT	TCACCGTCGA	CAAGATCCCG	GCGCAAAACC	550
R-24	GGGCGTTTAC	GGCCGTAATT	TCACCGTCGA	TAAACTTCCG	GCTCAGAACC	550
l-chi	TGACGCACCT	GCTGTACGGC	TTTATCCCTA	TCTGTGGCGG	TGACGGCATC	600
m-chi	TGACCCACCT	GCTGTACGGC	TTTATCCCGA	TCTGCGGCGG	CAATGGCATC	600
R-24	TGACGCACCT	GCTGTACGGC	TTTATCCCTA	TCTGTGGCGG	TGACGGCATC	600
l-chi	AACGACAGCC	TGAAAGAGAT	CGAAGGCAGC	TTCCAGGCGT	TACAGCGTTC	650
m-chi	AACGACAGCC	TGAAAGAGAT	TGAAGGCAGC	TTCCAGGCGT	TACAGCGCTC	650
R-24	AACGACAGCC	TGAAAGAGAT	TGAAGGCAGC	TTCCAGGCGT	TACAGCGCTC	650

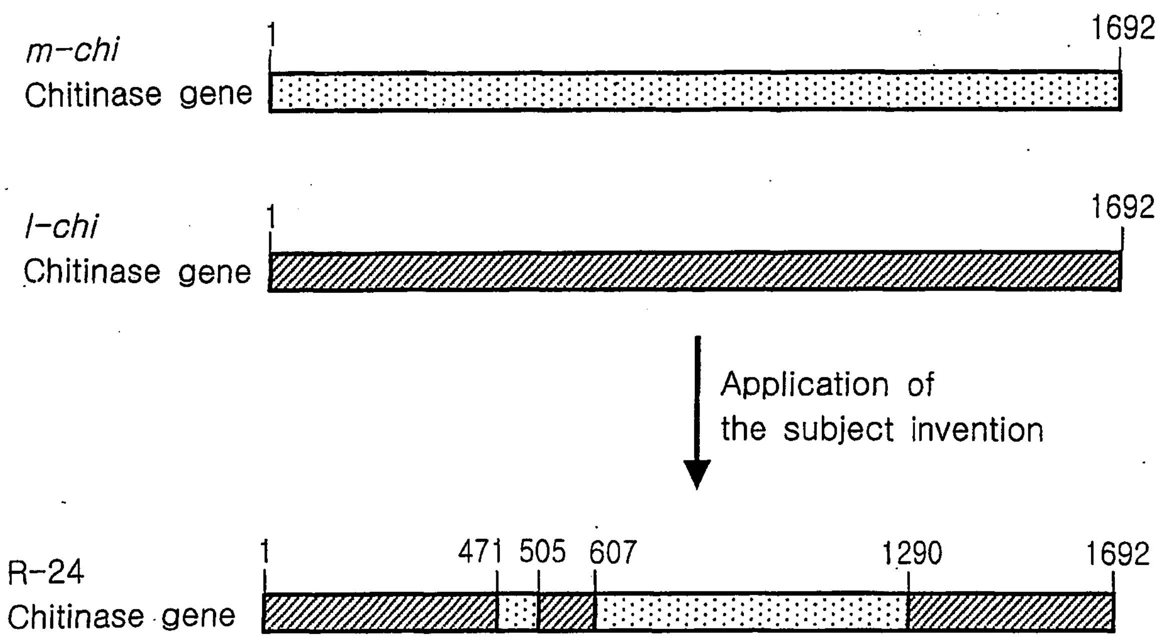
FIG. 8(continued)

l-chi	CTGTCAGGGG	CGTGAAGACT	TTAAGGTATC	GATCCACGAT	CCGTTCGCTG	700
m-chi	CTGCCAGGGC	CGCGAGGACT	TCAAAGTCTC	GGTCCACGAT	CCGTTCGCCG	700
R-24	CTGCCAGGGC	CGCGAGGACT	TCAAAGTCTC	GGTCCACGAT	CCGTTCGCCG	700
l-chi	CGCTGCAGAA	AGGTCAGAAG	GGCGTGACCG	CCTGGGACGA	CCCCTACAAA	750
m-chi	CGCTGCAAAA	AGCGCAGAAG	GGCGTGACCG	CCTGGGATGA	CCCCTACAAG	750
R-24	CGCTGCAAAA	AGCGCAGAAG	GGCGTGACCG	CCTGGGATGA	CCCCTACAAG	750
l-chi	GGCAACTTCG	GCCAGTTGAT	GGCGTTGAAA	CAGGCGCGCC	CGGACCTGAA	800
m-chi	GGCAACTTCG	GCCAGCTGAT	GGCGCTGAAG	CAGGCGCATC	CTGACCTGAA	800
R-24	GGCAACTTCG	GCCAGCTGAT	GGCGCTGAAG	CAGGCGCATC	CTGACCTGAA	800
l-chi	AATCCTGCCG	TCGATCGGTG	GCTGGACGTT	ATCCGATCCG	TTCTTCTTTA	850
m-chi	AATCCTGCCG	TCGATCGGCG	GCTGGACGCT	GTCCGACCCG	TTCTTCTTCA	850
R-24	AATCCTGCCG	TCGATCGGCG	GCTGGACGCT	GTCCGACCCG	TTCTTCTTCA	850
l-chi	TGGGCGATAA	GGTGAAGCGC	GATCGCTTCG	TCGGCTCGGT	GAAGGAGTTC	900
m-chi	TGGGCGACAA	GGTGAAGCGC	GATCGCTTCG	TCGGTTCGGT	GAAAGAGTTC	900
R-24	TGGGCGACAA	GGTGAAGCGC	GATCGCTTCG	TCGGTTCGGT	GAAAGAGTTC	900
l-chi	CTGCAAACCT	GGAAGTTCTT	TGATGGCGTA	GATATCGACT	GGGAATTCCC	950
m-chi	CTGCAGACCT	GGAAGTTCTT	CGACGGCGTG	GATATCGACT	GGGAGTTCCC	950
R-24	CTGCAGACCT	GGAAGTTCTT	CGACGGCGTG	GATATCGACT	GGGAGTTCCC	950
l-chi	GGGCGGGCAG	GGTGCTAACC	CGAAACTGGG	CAGTACGCAG	GATGGGGCAA	1000
m-chi	GGGCGGCAAA	GGCGCCAACC	CTAACCTGGG	CAGCCCGCAA	GACGGGGAAA	1000
R-24	GGGCGGCAAA	GGCGCCAACC	CTAACCTGGG	CAGCCCGCAA	GACGGGGAAA	1000
l-chi	CCTATGTGCA	GCTGATGAAA	GAGCTGCGCG	CCATGCTGGA	TCAGCTTTCG	1050
m-chi	CCTATGTGTT	GCTGATGAAG	GAGCTGCGGG	CGATGCTGGA	TCAGCTGTCG	1050
R-24	CCTATGTGTT	GCTGATGAAG	GAGCTGCGGG	CGATGCTGGA	TCAGCTGTCG	1050
l-chi	GCGGAAACGG	GCCGTAAGTA	TGAACTGACC	TCTGCGATCA	GCGCCGGCAA	1100
m-chi	GCGGAAACCG	GCCGCAAGTA	TGAGCTGACC	TCCGCCATCA	GCGCCGGTAA	1100
R-24	GCGGAAACCG	GCCGCAAGTA	TGAGCTGACC	TCCGCCATCA	GCGCCGGTAA	1100
l-chi	GGATAAAATC	GATAAGGTGG	ATTACAACAC	CGCACAAAAC	TCGATGGATC	1150
m-chi	GGACAAGATC	GACAAGGTGG	CTTACAACGT	TGCGCAGAAC	TCGATGGATC	1150
R-24	GGACAAGATC	GACAAGGTGG	CTTACAACGT	TGCGCAGAAC	TCGATGGATC	1150
l-chi	ACATTTTCCT	GATGAGTTAC	GACTTCTATG	GGGCATTCGA	TCTGAAAAAT	1200
m-chi	ACATCTTCCT	GATGAGCTAC	GACTTCTATG	GCGCCTTCGA	TCTGAAGAAC	1200
R-24	ACATCTTCCT	GATGAGCTAC	GACTTCTATG	GCGCCTTCGA	TCTGAAGAAC	1200
l-chi	CTGGGCCACC	AGACTGCGCT	GAAAGCGCCG	GCCTGGAAAC	CGGATACGGC	1250
m-chi	CTGGGGCATC	AGACCGCGCT	GAATGCGCCG	GCCTGGAAGC	CGGACACCGC	1250
R-24	CTGGGGCATC	AGACCGCGCT	GAATGCGCCG	GCCTGGAAGC	CGGACACCGC	1250
l-chi	GTATACCACG	GTGAATGGCG	TTAATGCACT	GCTCACGCAG	GGCGTGAAGC	1300
m-chi	TTACACCACG	GTGAACGGCG	TCAATGCGCT	GCTGGCGCAG	GGCGTCAAGC	1300
R-241	TTACACCACG	GTGAACGGCG	TCAATGCGCT	GCTGGCGCAG	GGCGTGAAGC	1300

FIG. 8 (continued)

l-chi	CGGGCAAAAT	CGTGGTGGGC	ACCGCCATGT	ACGGTCGCGG	TTGGACCGGG	1350
m-chi	CGGGCAAGAT	CGTGGTCGGC	ACCGCCATGT	ATGGCCGCGG	CTGGACCGGG	1350
R-24	CGGGCAAAAT	CGTGGTGGGC	ACCGCCATGT	ACGGTCGCGG	TTGGACCGGG	1350
l-chi	GTGAACGGTT	ACCAGAACAA	CATTCCGTTT	ACCGGCACCG	CCACTGGCCC	1400
m-chi	GTGAACGGCT	ACCAGAACAA	CATTCCGTTc	ACCGGTACCG	CCACTGGGCC	1400
R-24	GTGAACGGTT	ACCAGAACAA	CATTCCGTTT	ACCGGCACCG	CCACTGGCCC	1400
l-chi	GGTGAAAGGC	ACCTGGGAAA	ATGGCATCGT	GGATTACCGC	CAGATCGCCA	1450
m-chi	GGTTAAAGGC	ACCTGGGAGA	ACGGCATCGT	GGACTACCGC	CAAATCGCCG	1450
R-24	GGTGAAAGGC	ACCTGGGAAA	ATGGCATCGT	GGATTACCGC	CAGATCGCCA	1450
l-chi	ATGAGTTTAT	GAGCGGCGAA	TGGCAGTACA	GCTACGATGC	TACCGCTGAA	1500
m-chi	GCCAGTTCAT	GAGCGGCGAG	TGGCAGTATA	CCTACGACGC	CACGGCGGAA	1500
R-24	ATGAGTTTAT	GAGCGGCGAA	TGGCAGTACA	GCTACGATGC	TACCGCTGAA	1500
l-chi	GCACCCTATG	TCTTCAAACC	TTCCACTGGC	GATCTGATCA	CCTTCGACGA	1550
m-chi	GCGCCTTACG	TGTTCAAACC	TTCCACCGGC	GATCTGATCA	CCTTCGACGA	1550
R-24	GCACCCTATG	TCTTCAAACC	TTCCACTGGC	GATCTGATCA	CCTTCGACGA	1550
l-chi	TGCGCGCTCG	GTGCAGGCGA	AGGGCAAATA	TGTGCTGGAT	AAGCAGCTGG	1600
m-chi	TGCCCGCTCG	GTGCAGGCCA	AAGGCAAGTA	CGTGCTGGAT	AAGCAGCTGG	1600
R-24	TGCGCGCTCG	GTGCAGGCGA	AGGGCAAATA	TGTGCTGGAT	AAGCAGCTGG	1600
l-chi	GCGGGTTGTT	CTCATGGGAA	ATTGACGCCG	ACAACGGCGA	TATTCTGAAT	1650
m-chi	GCGGCCTGTT	CTCCTGGGAG	ATCGACGCGG	ATAACGGCGA	TATTCTCAAC	1650
R-24	GCGGGTTGTT	CTCATGGGAA	ATTGACGCCG	ACAACGGCGA	TATTCTGAAT	1650
l-chi	AACATGAACA	GCAGCCTGGG	CAACAGCGTC	GGTACGCCTT	AA	1692
m-chi	AGCATGAACG	CCAGCCTGGG	CAACAGCGCC	GGCGTTCAAT	AA	1692
R-24	AACATGAACA	GCAGCCTGGG	CAACAGCGTC	GGTACGCCTT	AA	1692

FIG. 9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Chitinase gene
1
1692
l-chi
Chitinase gene
1
1692
Application of
the subject invention
R-24
Chitinase gene
1
471
505
607
1290
1692

FIG. 10

```
Wild  ATGAATGGAAAAAGA AAAATTTTCACATGT ATTTCTATTGTAGGA ATCGGACTAGCTAGT  60
M-13  ATGAATGGAAAAAGA AAAATTTTCACATGT ATTTCTATTGTAGGA ATCGGACTAGCTAGC  60
M-20  ATGAATGGAAAAAGA AAAATTTTCACGTGT ATTTCTATTGTAGGA ATCGGACTAGCTAGT  60

Wild  TTTTCTAATTCTAGT TTCGCAGCAAGTGTA ACGGACAATTCAGTA CAAAATTCTATTCCC  120
M-13  TTTTCTAATTCTAGT TTCGCAGCAAGTGTA ACGGACAATTCAGTA CAAAATTCTATTCCC  120
M-20  TTTTCTAATCCTAGT TTCGCAGCAAGTGTA ACGGACAATTCAGTA CAAAATTCTATTCCC  120

Wild  GTAGTTAATCAACAA GTAGCTGCTGCAAAG GAAATGAAACCATTT CCGCAGCAAGTTAAT  180
M-13  GTAGTTAATCAACAA GTAGCTGCTGCAAAG GAAATGAAACCATTT CCGCAGCAAGTTTAT  180
M-20  GTAGTTAATCAACAA GTAGCTGCTGCAAAG GAAATGAAACCATTT CCGCAGCAAGTTAAT  180

Wild  TATGCAGGTGTTATA AAACCGAATCATGTT ACACAGGAAAGTTTA AATGCTTCTGTAAGA  240
M-13  TATGCAGGTGTTATA AAACCGAATCATGTT ACACAGGAAAGTTTA AATGCTTCTGTAAGA  240
M-20  TATGCAGGTGTTATA AAACCGAATCATGTT ACACAGGAAAGTTTA AATGCTTCTGTAAGA  240

Wild  AGTTACTACGATAAT TGGAAAAAGAAATAT TTGAAAAATGATTTA TCTTCTTTACCTGGT  300
M-13  AGTTACTACGATAAT TGGAAAAAGAAATAT TTGAAAAATGATTTA TCTTCTTTACCTGGT  300
M-20  AGTTACTACGATAAT TGGAAAAAGAAATAT TTGAAAAATGATTTA TCTTCTTTACCTGGT  300

Wild  GGTTATTATGTAAAA GGAGAGATTACAGGT GATGCTGATGGGTTT AAGCCACTTGGAACT  360
M-13  GGTTATTATGTAAAA GGAGATATTACAGGT GATGCTGATGGGTTT AAGCCACTTGGAACT  360
M-20  GGTTATTATGTAAAA GGAGATATTACAGGT GATGCTGATGGGTTT AAGCCACTTGGAACT  360

Wild  TCAGAAGGTCAAGGG TATGGGATGATAATT ACAGTATTAATGGCT GGTTATGATTCGAAT  420
M-13  TCAGAAGGTCAAGGG TATGGGATGATAATT ACAGTATTAATGGCT GGTTATGATTCGAAT  420
M-20  TCAGAAGGTCAAGGG TATGGGATGATAATT ACAGTATTAATGGCT GGTTATGATTCGAAT  420

Wild  GCTCAAAAAATCTAT GACGGTTTATTTAAA ACAGCAAGAACTTTT AAAAGTTCTCAAAAT  480
M-13  GCTCAAAAGATCTAT GACGGTTTATTTAAA ACAGCAAGAACTTTT AAAAGTTCTCGAAAT  480
M-20  GCTCAAAAGATCTAT GACGGTTTATTTAAA ACAGCAAGAACTTTT AAAAGTTCTCGAAAT  480

Wild  CCTAATTTAATGGGA TGGGTTGTCGCAGAT AGTAAAAAAGCACAA GGTCATTTTGATTCT  540
M-13  CCTAATTTAATGGGA TGGGTTGTCGCAGAT AGTAAAAAAGCACAA GGTCATTTTGATTCT  540
M-20  CCTAATTTAATGGGA TGGGTTGTCGCAGAT AGTAAAAAAGCACAA GGTCATTTTGATTCT  540

Wild  GCTACTGATGGAGAT TTAGATATTGCGTAT TCTCTTCTTCTTGCT CATAAGCAGTGGGGA  600
M-13  GCTACTGATGGAGAT TTAGATATTGCGTAT TCTCTTCTTCTTGCT CATAAGCAGTGGGGA  600
M-20  GCTACTGATGGAGAT TTAGATATTGCGTAT TCTCTTCTTCTTGCT CATAAGCAGTGGGGA  600

Wild  TCTAATGGAACAGTG AATTATTTGAAAGAA GCACAAGACATGATT ACAAAAGGTATTAAA  660
M-13  TCTAATGGAACAGTG AATTATTTGAAAGAA GCACAAGACATGATT ACAAAAGGTATTAAA  660
M-20  TCTAATGGAACAGTG AATTATTTGAAAGAA GCACAAGACATGATT ACAAAAGGTATTAAA  660

Wild  GCTAGTAATGTTACA AATAATAACCGACTA AATTTAGGCGATTGG GATTCTAAAAGTTCA  720
M-13  GCTAGTAATGTTACC AATAATACCCGACTA AATTTAGGCGATTGG GATTCTAAAAGTTCA  720
M-20  GCTAGTAATGTTACA AATAATAACCGACTA AATTTAGGCGATTGG GATTCTAAAAGTTCA  720
```

FIG. 10 (continued)

```
Wild  CTTGATACGAGACCA TCTGATTGGATGATG TCACACCTTAGAGCA TTTTATGAATTTACA  780
M-13  CTTGATACGAGACCA TCTGATTGGATGATG TCACACCTTAGAGCA TTTTATGAATTTACA  780
M-20  CTTGATACGAGACCA TCTGATTGGATGATG TCACACCTTAGAGCA TTTTATGAGTTTACA  780

Wild  GGTGATAAAACTTGG CTTACTGTTATTAAT AATTTGTACGATGTT TATACGCAATTTAGT  840
M-13  GGTGATAAAACTTGG CTTACTGTTATTAAT AATTTGTACGATGTT TATACGCAGTTTAGT  840
M-20  GGTGATAAAACTTGG CTTACTGTTATTAAT AATTTGTACGATGTT TATACGCAATTTAGT  840

Wild  AATAAGTACTCTCCA AATACAGGACTTATT TCAGATTTCGTTGTA AAAAACCCACCACAA  900
M-13  AATAAGTACTCTCCA AATACAGGACTTATT TCAGATTTCGTTGTA AAAAACCCACCACAA  900
M-20  AATAAGTACTCTCCA GATACAGGACTTATT TCAGATTTCGTTGTA AAAAACCCACCACAA  900

Wild  CCCGCACCTAAAGAC TTCTTAGAGGAGTCA GAATATACAAATGCA TATTATTACAATGCT  960
M-13  CCCGCACCTAAAGGC TTCTTAGGGGAGTCA GAATATACAAATGCA TATTATTACAATGCT  960
M-20  CCCGCACCTAAAGGC TTCTTAGGGGAGTCA GAATATACAAATGCA TATTATTACAATGCT  960

Wild  AGTCGGGTACCATTG AGAATTGTAATGGAC TATGCGATGTACGGC GAGAAAAGAAGTAAA  1020
M-13  AGTCGGGTACCATTG AGAATTGTAATGGAC TATGCGATGTACGGC GAGAAAAGAAGTAAA  1020
M-20  AGTCGGGTACCATTG AGAATTGTAATGGAC TATGCGATGTACGGC GAGAAAAGAAGTAAA  1020

Wild  GTCATTTCTGATAAA GTTTCTTCGTGGATT CAAAATAAAACGAAT GGAAATCCTTCTAAA  1080
M-13  GTCATTTCTGATAAG GTTTCTTCGTGGATT CAAAATAAAACGAAT GGAAATCCTTCTAAA  1080
M-20  GTCATTTCTGATAAG GTTTCTTCGTGGATT CAAAATAAAACGAAT GGAGATCCTTCTAAA  1080

Wild  ATTGTGGATGGTTAT CAATTAAATGGATCT AATATTGGTAGTTAT TCAACTGCTGTATTT  1140
M-13  ATTGTGGATGGTTAT CAATTAGATGGATCT AATATTGGTAGTTAT CCAACTGCTGTATTT  1140
M-20  ATTGTGGATGGTTAT CAATTAGATGGATCT GATATTGGTAGTTAT TCAACTGCTGTATTT  1140

Wild  GTTTCACCGTTTATT GCTGCAAGTATAACA AGTAGCAATAATCAA AAGTGGGTAAATAGC  1200
M-13  GTTTCACCGCTTATT GCTGCAAGTACAACA AGTAGCAATAATCAA AAGTGGGTAAATAGC  1200
M-20  GTTTCACCGTTTATT GCTGCAAGTATAACA AGTAGCAATAATCAA AAGTGGGTAAATAGC  1200

Wild  GGTTGGGATTGGATG AAGAATAAGAGAGAA AGTTATTTTAGTGAT AGTTATAATTTATTA  1260
M-13  GGTTGGGATTGGATG AAGAATAAGAGAGAA AGTTATTTTAGTGAT AGTTATAATTTATTA  1260
M-20  GGTTGGGATTGGATG AAGAATAAGAGAGAA AGTTATTTTAGCGAT AGTTATAATTTGTTA  1260

Wild  ACTATGTTATTCATT ACAGGAAATTGGTGG AAACCTGTACCTGAT GATACAAAAATACAA  1320
M-13  ACTATGTTATTCATT ACAGGGAATTGGTGG AAACCCGTACCTGGT GATACAAAAATACAA  1320
M-20  ACTATGTTATTCATT ACGGGAAATTGGTGG AAACCTGTACCTGAT GATACAAAAATACAA  1320

Wild  AATCAAATAAATGAT GCAATTTATGAAGGA TACGATAATTAA  1362
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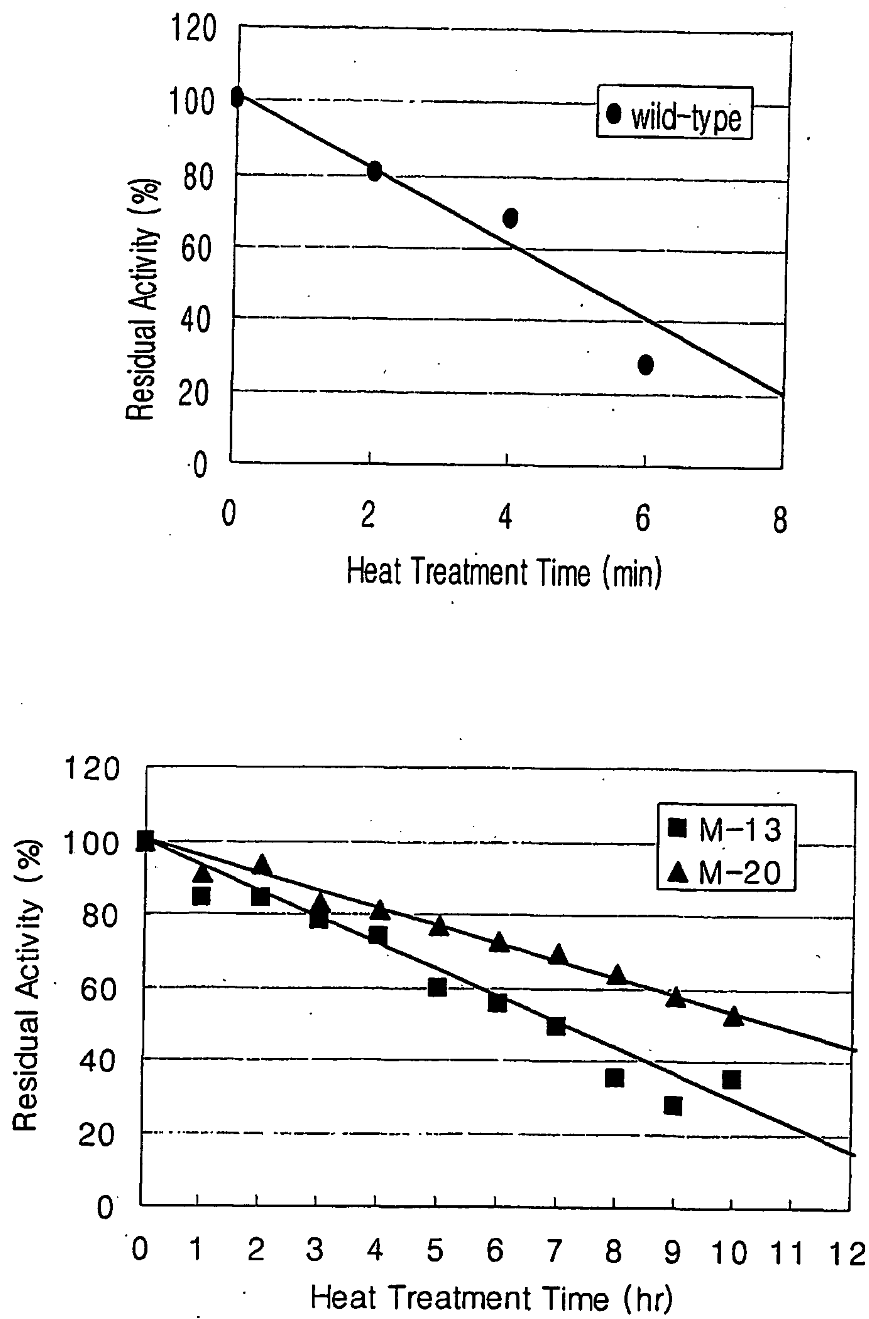
FIG. 11



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80
60
40
20
0
Residual Activity (%)
wild-type
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2
4
6
8
Heat Treatment Time (min)
120
100
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60
40
20
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Residual Activity (%)
M-13
M-20
0 1 2 3 4 5 6 7 8 9 10 11 12
Heat Treatment Time (hr)

SEQUENCE LISTING

<110> Amicogen, Inc.

<120> Method for generating recombinant DNA library using unidirectional single-stranded DNA fragments

<150> KR 2000-66889
<151> 2000-11-10

<160> 27

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<400> 8

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<223> Chitinase recombinant DNA 8

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01031

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
C12N 15/10, 15/00 : C12P 19/34 : C12Q 1/68 : G01N 33/566

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched
Korean Patents and Applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
Delphion, PAJ, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,994,368 (Syntex Inc.) 19 Feb 1991 See the abstract	1-17
A	US 5,811,238 (Affymax Technologies N.V.) 22 Sep 1998 See the abstract & Figures	1-17
A	US 5,962,272 (Clontech Lab. Inc.) 05 Oct 1999 See the abstract & Figures	1-17

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 OCTOBER 2001 (17.10.2001)	18 OCTOBER 2001 (18.10.2001)
Name and mailing address of the ISA/KR Korean Intellectual Property Office Facsimile No.	Authorized officer AHN, Mi-Chung Telephone No.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR01/01031

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		US 5273879 A	28 DEC 1993
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1995			
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		WO 9724456 A2	10 JUL 1997
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